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Research paper

Using recombination-dependent lethal mutations to stabilize reporter flaviviruses for rapid serodiagnosis and drug discovery

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ABSTRACT

Background: Many flaviviruses are significant human pathogens that cause global public health threats. Developing research tools for studying and diagnosing these pathogens is a top priority. Reporter flaviviruses are useful tools for studying viral pathogenesis, diagnosing disease, and screening antiviral compounds. However, the stability of reporter flaviviruses has been challenged by viral RNA recombination, leading to deletion of the engineered reporter gene during viral replication. The instability of reporter viruses has limited their application to research and countermeasure development. Thus, new approaches to overcome the instability of reporter flaviviruses are critically needed to advance the flavivirus field.

Methods: To create a stable flavivirus bearing a reporter gene, we engineered mutations in the viral capsid gene that are rendered virus-lethal upon recombination. Thus, only non-recombined reporter virus propagates. We tested this strategy using Zika virus (ZIKV) bearing a nano-luciferase (NanoLuc) gene and passaged both virus with capsid mutations and virus without mutations.

Findings: The recombination-dependent lethal mutations succeeded in stabilizing the NanoLuc ZIKV through ten passages, while WT reporter virus showed instability as early as five passages. The stability of NanoLuc ZIKV was supported by RT-PCR, sequencing, focus forming assay, and luciferase assay. The success of this method was reconfirmed by also establishing a stable NanoLuc Yellow Fever 17D virus, indicating that the recombination-dependent lethal approach can be applied to other flaviviruses. To demonstrate the utility of the stable reporter viruses, we showed that NanoLuc ZIKV and YFV17D could be used to measure neutralizing antibody titers with a turnaround time as short as four hours. Importantly, the neutralizing antibody titers derived from the reporter virus assay were equivalent to those derived from the conventional plaque assay, indicating the new assay maintains the gold standard of serology testing. Furthermore, using a known inhibitor, we showed that the reporter viruses could be reliably used for antiviral evaluation.

Interpretation: The study has developed a recombination-dependent lethal approach to produce stable reporter flaviviruses that may be used for rapid serodiagnosis, trans-gene delivery, vaccine evaluation, and antiviral discovery.

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1. Introduction

Flaviviruses are an important class of medically relevant, arthropod-transmitted viruses that include the widespread four serotypes of dengue virus (DENV) [1], the neurotropic West Nile virus (WNV) and Japanese encephalitis virus (JEV), the prototypical yellow fever virus (YFV), and the recently expanded Zika virus (ZIKV) [2]. Though much research has elucidated pathogenic mechanisms for these viruses, approved antiviral countermeasures are limited to vaccines for YFV, JEV, and DENV, with much room for improvement for these and other pre-clinical vaccines [3]. Direct-acting antiviral compounds remain elusive for all flaviviruses,

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Research in context

Evidence before this study

Reporter viruses are valuable research tools in areas including pathogenesis studies, drug screenings, and disease diagnosis. For the medically relevant flaviviruses, including the serotypes of dengue virus, yellow fever virus, and Zika virus, the utility of these molecular tools has been limited by loss of the reporter gene during longer growth periods.

Added value of this study

We have solved this problem for Zika and yellow fever reporter viruses by addition of recombination-dependent lethal mutations that stop viral spread if the reporter gene is removed. We showed that these viruses can be grown successively on cells more than ten times without loss of the reporter gene. We then used these viruses to determine the neutralizing antibody levels in mouse and human sera and compared the results with traditional methods, with singular agreement between the outcomes.

Implication of all the available evidence

These assays, which are critical for vaccine clinical trials and also used for disease diagnosis, can be accomplished in as little as four hours, which is a significant time improvement over current methods.

notwithstanding a concerted effort from pharmaceutical companies and academic researchers [4,5].

Reverse genetic systems for these human pathogens have been invaluable for the development and testing of a new generation of vaccines [6−10] as well as for screening for new antiviral compounds and implementing improved diagnostics [11,12]. The latter two of these functions have particularly benefited from the advance of reporter viruses as molecular tools. The earliest flavivirus reporter constructs consisted of a reporter gene engineered in place of most of the viral structural genes [13]. These replicons could be trans-complemented with the structural genes to form single-round infectious particles or integrated to make a replicon-producing cell line [12,14,15]. Though useful, replicons do not complete a full viral life cycle, only mimicking viral translation and replication. Second generation, fully infectious, reporter flaviviruses were made by inserting an internal ribosome entry site (IRES) followed by the reporter gene in a permissible site in the 3′ UTR of a full-length cDNA clone [16−18]. While successful in the short term, IRES reporter viruses grown for any length of time quickly lose the reporter gene due to deletions in the reporter gene [17]. Alternatively, a different, and more robust method, for reporter gene insertion was developed using YFV. The reporter gene was inserted at the junction between the 5′ UTR and the capsid gene, but due to RNA regulatory elements that are continuous from the 5′ UTR into the capsid, a portion of the capsid must be duplicated upstream of the reporter gene. The YFV construct was made with 25 capsid amino acids duplicated [19]. This method for fashioning reporter flaviviruses is considerably more stable than IRES driven constructs, with successful stability typically reported to four or five passages. However, despite codon optimization in the capsid gene (to reduce homology), recombination will still result in a non-reporter virus after extended passaging [20].

Reporter virus stability (defined by how long it can be grown on cells and maintain an intact, functional reporter gene) is vital for long-term pathogenesis studies that rely on reporter gene output. As arboviruses, flaviviruses cycle between mammalian and insect hosts. Experiments that capture this complicated life cycle could be simplified by the use of reporter viruses, though the long-term nature of these investigations would require these viruses to be exceptionally stable. Additionally, diagnostic and industrial use of reporter flaviviruses for large-scale drug screens and serology assays will require

stiff confidence in the reporter output and how it relates to viral titers. To answer these unmet needs, we developed a method of stabilizing reporter flaviviruses to greater than ten passages.

The first Zika reporter virus was published alongside the first cDNA clone [21]. While its stability was not reported, other, similar ZIKV constructs have been made and their stability after passaging reported [20,22,23]. None have been reported as stable beyond four or five passages in cell culture, with the exception of a Zika reporter virus recently published this year [24]. Their work compares different lengths of duplicated capsid and the effect thereof on viral replication. This testing, coupled with a frameshift mutation in the duplicated portion, are sufficient to stabilize their reporter virus, with the frameshift mutation disrupting correct protein translation if recombination occurs. Here, we have developed a strategy to stabilize reporter flaviviruses, using recombination-dependent lethal mutations in the duplicated capsid portion to block virion formation upon recombination. We showed this strategy to be effective for stabilizing both a NanoLuc ZIKV and YFV to greater than ten passages and demonstrated these stabilized viruses' effectiveness in serology and antiviral assays.

2. Materials and methods

2.1. Viruses and cells

Zika virus strain Dakar 41525 and YF17D strain YFS11 were cloned into full-length plasmids using the low copy pCC1 vector as has been previously described [6,21]. The NanoLuc gene and capsid mutations for ZIKV were inserted using an overlap PCR, restriction digest, and ligation strategy. Gene insertions and mutations were cloned in YFV using NEBuilder HiFi DNA Assembly mix (NEB E2621). Viruses were recovered after electroporation (Biorad GenePulser Xcell) of *in vitro* transcribed RNAs in Vero (ATCC Cat# CCL-81, RRID: CVCL 0059) cells as previously described [21]. All Vero cells were grown in Dulbecco's Modified Eagle Medium (Gibco 11965) supplemented with 10% fetal bovine serum (FBS, Hyclone SH30071) and 1% penicillin/streptomycin (Gibco 15140). Huh7 cells (RRID: CVCL 0336) were grown in Dulbecco's Modified Eagle Medium with Glutamax (Gibco 10566) supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% non-essential amino acids (Gibco 11140). Infections were carried out in the same media excepting supplementation with 2% fetal bovine serum instead of 10%. Cells were grown at 37 °C in a humidified incubator with 5% CO_2.

2.2. Capsid mutation screens

This experiment was carried out as detailed in [25]. Briefly, *in vitro* transcribed RNAs were electroporated into Vero cells and plated in 24-well plates. Cells electroporated with no RNAs were used as a negative control (mock). At 4, 24, 48, 72, and 96 h, the supernatants were harvested, and the cells were washed with phosphate buffered saline (PBS, Gibco 10010023), lysed with Cell Culture Lysis Reagent (Promega E153A), and frozen at −80 °C. The supernatants were used to infect fresh Vero cells, which were washed and lysed at 24 h. Lysed cells were moved to a 96 well plate and read for luciferase activity after addition of NanoGlo substrate (Promega N1150) using a Biotek Cytation 5 plate reader according to the manufacturer's recommendation.

2.3. Immunofluorescence assay

Vero cells were aliquoted into chamber slides post-electroporation. At the indicated time points, cells were washed with PBS and fixed with cold methanol, covered, and placed at −30 °C for >30 min. Slides were then washed with PBS and blocked with PBS+1% FBS overnight at 4 °C. The pan-flavivirus envelope antibody 4G2 (ATCC

Cat# HB-112, RRID: CVCL J890) was used to probe for infected cells. A secondary goat anti-mouse IgG antibody conjugated with Alexa Fluor 488 (Thermo Fisher Scientific Cat# A-11001, RRID: AB 2534069) was then used to probe for 4G2. Slides were stained with DAPI (Vector Laboratories, H-1200) and then imaged on a Nikon Eclipse Ti2 microscope. ImageJ (NIH) was used to process these images.

2.4. Focus forming assay

All viruses were titered using a focus-forming assay. Viruses were serially diluted ten-fold and used to infect Vero cells that had been seeded the day previously at 2×10^5 cells per well in a 24-well plate. After a 1-h infection, the inoculum was removed and methylcellulose and DMEM was overlaid. At four days post infection, the overlay was removed, and cells were fixed with a 1:1 solution of methanol/acetone for >15 min. Plates were washed with PBS 3X, blocked with PBS +3% FBS, and then incubated with virus-specific mouse immune ascites fluid (MIAF, World Reference Center for Emerging Viruses and Arboviruses, UTMB). After >1-h incubation with MIAF, plates were washed and incubated with a horseradish peroxidase-conjugated anti-mouse IgG antibody (SeraCare KPL Cat# 474-1806, RRID: AB 2307348). After a 3X PBS wash, foci were developed using an AEC peroxidase substrate kit (Enzo 43825) according to the manufacturer's protocol. Images were acquired with a BioRad ChemiDoc Imaging System.

2.5. Growth curve

Vero cells were seeded at 8×10^5 cells per well in a six well plate the day before infection. Cells were infected at a MOI of 0.01 for 1 h followed by a 3X PBS wash and addition of media supplemented with 2% FBS. Cell supernatant samples were taken at 24, 48, 72, 96, and 120 h and titered by focus-forming assay. Samples at 24 h were directly used for focus-forming assay, while samples taken at 48−120 h were first diluted 10-fold, thus giving a ten-fold difference in the limit of detection.

2.6. Luciferase assay

Vero cells were seeded in an opaque, white 96 well plate at 1.5×10^4 cells per well the day before infection. Viruses were diluted to a MOI of 0.5 and added to plates after removal of media. At the indicated time points, the media was removed, and cells were washed 2X with PBS. NanoGlo substrate, diluted 1:50 in NanoGlo Assay Buffer, was then directly added to cells and plates were read on a BioTek Cytation 5 instrument after 3 min.

2.7. Reporter virus passaging and stability

Virus recovered after electroporation formed the P0 stock. 500 μL of this was added to a T75 flask seeded the day before with Vero cells. The infection continued until cell death was observed (3-day average for DK Nano and YF Nano, 4-day average for DK23 Nano and YF4 Nano) after which media was harvested and aliquoted. 500 μL of an aliquot was then used to infect a fresh T75 flask for a new passage. This was carried out in duplicate series for each virus. Stability was assessed by isolating viral RNA (Qiagen 52904) and using this for an RT-PCR reaction (Invitrogen 12574) with primers encompassing the 5′ UTR through the capsid. The products were then run on a 0.6% agarose gel to observe size.

2.8. Reporter PRNT assays

Reporter neutralization tests were done by serially diluting sera two-fold, starting at 1:50 in DMEM. Sera samples against ZIKV and DENV1-4 were pooled from mice infected with the respective virus. YFV and JEV sera samples were from mice vaccinated against the virus. Normal serum was pooled from mice that were naïve for flavivirus infection. Serially diluted sera samples were mixed 1:1 with the respective reporter virus and incubated at 37 °C for 1 h. The virus/sera mixture was then plated on Vero cells in a white 96 well plate that was seeded at 1.5×10^4 cells per well the day before. After a 4-h infection at 37 °C, the wells were washed 2X with PBS and 50 μL of NanoGlo substrate diluted 1:50 in NanoGlo Assay Buffer was added to the cells. Plates were read in a BioTek Cytation 5 plate reader after 3 min. Positive controls consisted of virus infection with no sera. Negative controls comprised virus plated in wells with no cells. This negative control allows for subtraction of background luciferase signals from the virus media. Results were graphed as a percent of positive control, with the negative control set as zero. The data was analyzed by four parameter nonlinear regression, with the top and bottom constrained to 100 and zero, respectively.

2.9. Plaque reduction neutralization tests

PRNT assays were done as previously published [30]. Briefly, 2-fold serially diluted sera samples were mixed 1:1 with virus equal to 200 plaque-forming units. After 1-h incubation at 37 °C, the mixture was placed on a confluent monolayer of Vero cells in a 6-well plate for 1 h. Afterwards, the inoculum was replaced with a methylcellulose overlay and the plates were further incubated until plaques became clear. The wells were then stained with crystal violet and plaques were counted.

2.10. Antiviral assays

The panflavivirus inhibitor NITD008 was two-fold serially diluted in 90% DMSO to a concentration starting at 10 μM. These were mixed with virus (MOI 0.01) and plated on Huh7 cells that were seeded at 1.5×10^4 cells per well the previous day. Cells were washed 48 h post infection three times with PBS followed by addition of NanoGlo substrate diluted 1:100 in NanoGlo Assay Buffer. Plates were read by a BioTek Cytation 5 plate reader after 3 min.

2.11. Statistical analysis

Graphpad Prism 8 was used for graphing and statistical analysis. Statistical tests used, as well as significance levels, are denoted in the figure legends. Instead of standard deviation, all replicated values are shown on each graph.

2.12. Illustrations

Figures were created using Biorender and Abode Illustrator.

3. Results

3.1. Recombination-dependent fatal mutations

Flavivirus reporter genes are commonly engineered at the beginning of the genome, after the 5′ UTR [11,19,21,26] (Fig. 1(a)). RNA regulatory elements are present in both the 5′ UTR and the capsid gene, (reviewed in [27,28]), thus the beginning of the capsid gene (25 amino acids) must be duplicated before the reporter gene in order to preserve viral translation and replication (see Fig. 1(a)). Duplicating this portion of the capsid greatly increases the chance of homology-mediated recombination, leading to elimination of the reporter gene. Reducing the homology by codon optimization of the full capsid gene improves stability but does not stop recombination (see Fig. 2(E), DK Nano, top panels). We explored a further way of stabilizing a reporter ZIKV (Dakar strain, DK). It is known
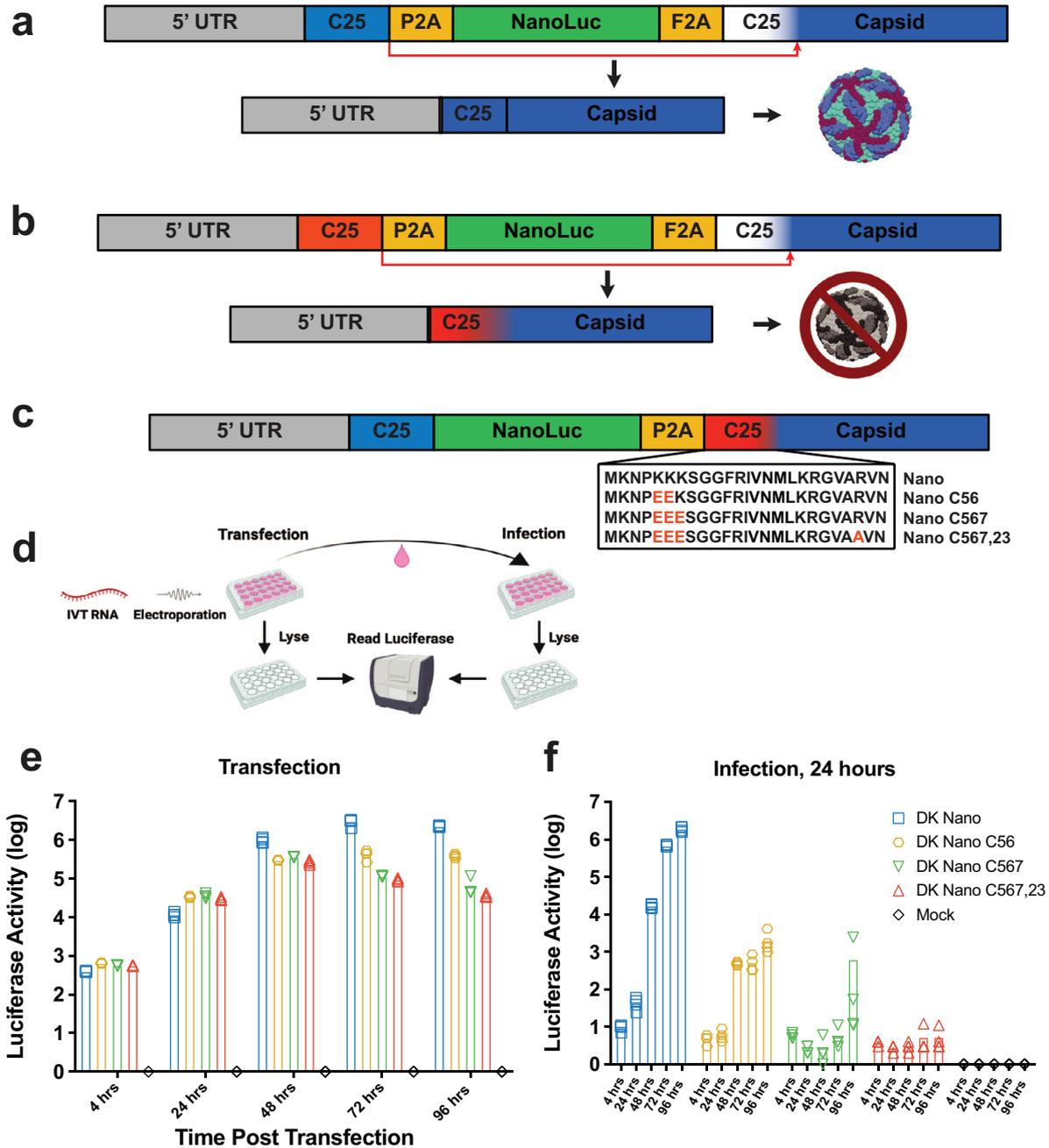
Fig. 1. Identification of recombination-dependent fatal mutations. (a) Scheme of reporter ZIKV based on the African lineage Dakar 41525 strain (DK). The nano luciferase gene (Nano) is eliminated by recombination, resulting in WT virus, despite codon optimization (denoted by white C25). (b) Scheme of reporter virus carrying recombination-dependent lethal mutations in the C25 (indicated in red). These mutations become lethal to viral particle formation upon recombination with the full capsid protein. (c) Genome scheme for deleterious capsid mutation screen. Basic amino acids in the beginning of the capsid protein were mutated to negative or neutral amino acids. (d) Experimental scheme to screen capsid mutations for effect on viral particle formation. *In vitro* transcribed RNAs of full-length reporter ZIKV with-and-without capsid mutations were electroporated on Vero cells and plated on 24 well plates (n = 4). At 4, 24, 48, 72, and 96 h post transfection, cell supernatants were collected for subsequent infection and cells were washed and lysed for luciferase measurement (transfection). The cell supernatants were used to infect naive Vero cells, which were all washed and lysed 24 h post infection after which luciferase readings were taken. Each virus was done in quadruplicate. (e) Luciferase readings from electroporated RNAs at the indicated time points. (f) Luciferase readings taken at 24 h post infection with supernatants collected at the time points indicated on the *X* axis from E. High luciferase activity indicates that high titers of infectious virus were made post electroporation, with results indicating charge-reversing mutations in the capsid inhibit viral particle formation.

that DENV requires a threshold of positive charges at the beginning of the capsid in order to form infectious viral particles [25]. We hypothesized that this information could provide a way to stabilize a reporter ZIKV. Charge reversing mutations, from positively to negatively charged amino acids, were designed in the C25 region that upon recombination would become part of the full capsid gene and lead to non-infectious viral particle formation (Fig. 1(b)). In this way, only non-recombined reporter viruses are passed on.

To test this strategy, we first engineered an increasing number of positive to negative charge mutations in the full capsid gene of a NanoLuc reporter ZIKV to find what was sufficient to stop viral particle formation (Fig. 1(c)). Fig. 1(D) details the experimental scheme. Luciferase levels assayed at 4, 24, 48, 72, and 96 h following transfection of *in vitro* transcribed RNAs indicate robust translation and replication for all viruses (Fig. 1(e)). The supernatants for each of these time points was used to infect fresh Vero cells which were assayed
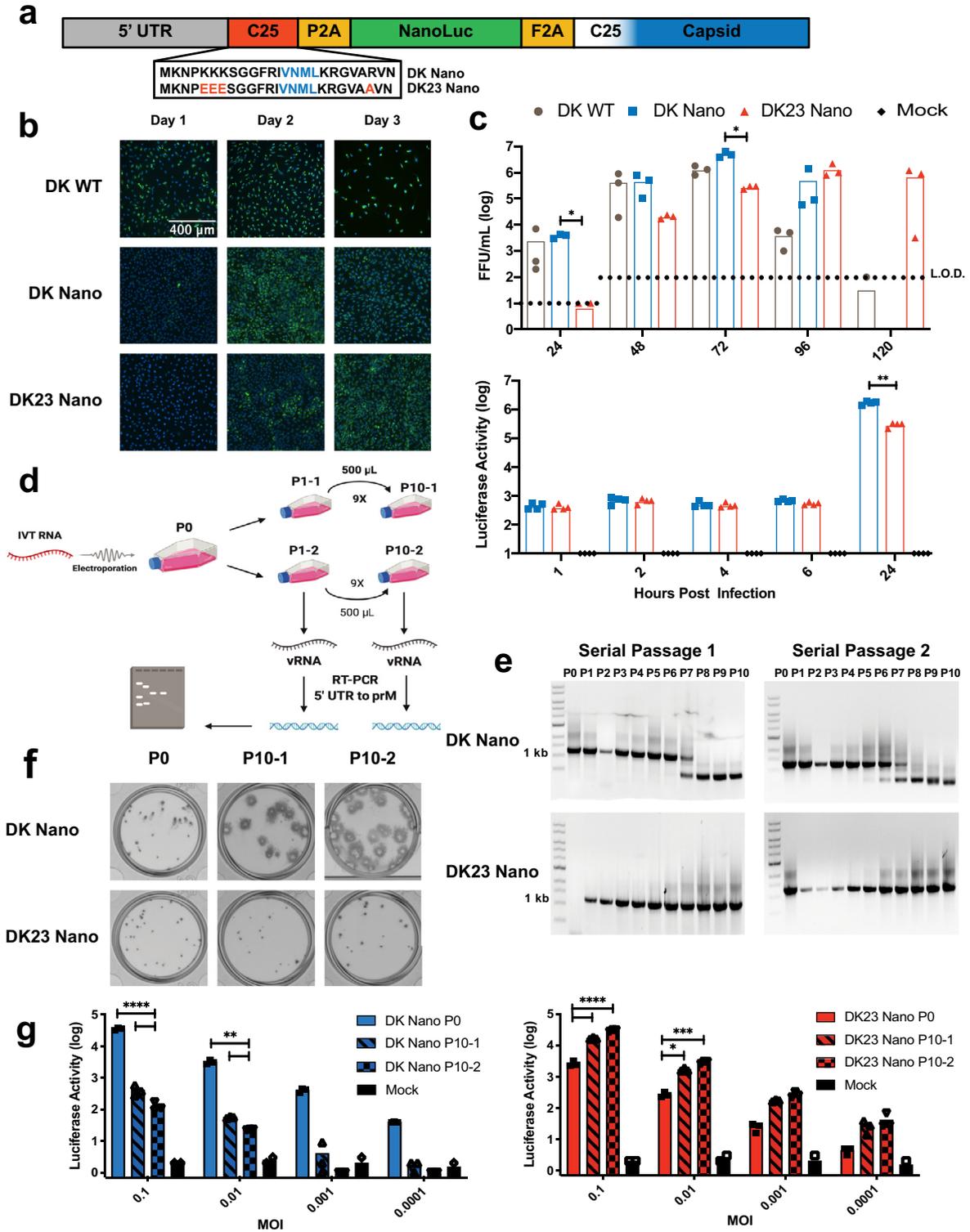
Fig. 2. A stable NanoLuc ZIKV. (a) Genome scheme of reporter ZIKV with and without capsid mutations. For reference, the amino acids corresponding to the 5′ cyclization sequence are highlighted in blue. (b) Comparison of WT Dakar strain ZIKV, DK Nano, and DK23 Nano IFA. Vero cells were seeded post-electroporation and fixed on days 1−3. Viral envelope protein was probed by 4G2 Ab. Scale is the same for all pictures. (c) Replication kinetics of the different viruses on Vero cells by both focus forming (MOI 0.01, $n = 3$) and luciferase assay (MOI 0.5, $n = 4$). Data comparison was done with 2-way ANOVA, using Tukey's post-hoc test for multiple comparisons (*$=p<0.05$, **$=p<0.0001$). (d) Virus passaging scheme. Virus collected post electroporation was termed P0 and 500 μL was used to inoculate a T75 flask of naive Vero cells. Infection proceeded until CPE was observed, $\sim$3 days, after which virus was harvested and 500 μL was passaged onto fresh Vero cells until P10 was reached. This was done in two independent passaging series. Viral RNA was harvested from each passage and RT-PCR performed from the 5′ UTR through the end of the capsid gene. Stability was observed by the size of the RT-PCR band compared to P0. (e) Passage results from two independent series P0 to P10 for DK Nano and DK23 Nano. RT-PCR band for full length reporter virus is 1225 bp. The WT, non-reporter virus product is 508 bp. DK Nano shows instability at P6 or P7, while DK23 Nano maintains a consistent RT-PCR product size through P10. (f) Focus sizes for DK Nano and DK23 Nano P0 and P10 after 4 days of infection on Vero cells. The P0 viruses formed tiny foci for both DK Nano and DK23 Nano viruses. However, the P10 DK Nano developed large foci, whereas P10 K23 Nano remained tiny foci. (g) Luciferase assay of P0 and P10 viruses. Vero cells were infected with indicated viruses at different MOIs. At 24 h post infection, intracellular luciferase activities were measured. Each time point was repeated 3 times, with each replicate shown. 2-way ANOVA with Tukey's post-hoc test was used to assess significance (*$=p<0.05$, **$=p<0.01$, ***$=p<0.001$, ****$=p<0.0001$).

for luciferase levels as a surrogate for infectious virus. The very low luciferase levels for DK Nano C567,23 (numbers represent the amino acid position in capsid protein) indicate that four capsid mutations are necessary to abrogate production of infectious virus (Fig. 1(f)).

3.2. Stable NanoLuc ZIKV

The four-amino acid capsid mutations (found to be necessary to stop virus formation) were engineered in the C25 region of a reporter ZIKV (designated DK23 Nano) and compared against a reporter ZIKV without capsid mutations (DK Nano) (Fig. 2(a)). Both reporter viruses show delayed growth and spread post-electroporation compared to DK WT (non-reporter) virus by immunofluorescence assay (IFA). The replication of DK23 Nano was further delayed compared to DK Nano; nevertheless, by day 3, 100% of cells were E protein positive (Fig. 2 (b)). Growth kinetics were assayed by both focus-forming (Fig. 2(c), top panel) and luciferase assay (bottom panel). Jointly, these experiments show that DK23 Nano was attenuated in replication when compared to DK Nano, but appreciable titers ($>10^6$ FFU/ml) and luciferase levels ($>10^5$ light units) were still attained. Both viruses were serially passaged ten times in duplicate as detailed in Fig. 2(d). Stability was assessed by observation of the size of the RT-PCR product from the 5′ UTR to the capsid. The RT-PCR product of DK Nano decreases in size at P6 or P7 (Fig. 2(e), top panels), while the DK23 Nano product is consistent through P10 (bottom panels). P10 RT-PCR products were sequenced, and results confirmed that DK23 Nano was unchanged while DK Nano had reverted to WT. Comparison of foci size between P0 and P10 showed consistent results from DK23 Nano, while DK Nano P10 foci resembled DK WT plaques (Compare Figs. 2(f) and S1a). Finally, luciferase assay carried out at multiple MOIs comparing P0 and P10 viruses showed that DK23 Nano P10 virus performed robustly, even significantly more robustly than the original P0 virus (Fig. 2(g), right panel). Sequencing the DK Nano and DK23 Nano P10-1 and P10-2 viruses showed minimal consensus level amino acid changes (Fig. S1b). In contrast, DK Nano showed considerably decreased luciferase activity (Fig. 2(g), left panel). Collectively, these data demonstrate that recombination-dependent lethal mutations were successful in stabilizing a NanoLuc reporter ZIKV to at least ten passages in cell culture.

3.3. Stable NanoLuc YFV

We next hypothesized that this strategy for stabilizing reporter ZIKV could be successful with another flavivirus, YFV. We followed a similar workflow with YFV as with ZIKV and first screened charge-reversing mutations in the full capsid gene of a YF17D NanoLuc virus (Fig. 3(a)). These mutations did not affect viral translation and replication after the genome-length RNAs were electroporated into cells (Fig. 3(b), left panel). Infection outcomes indicated that, similar to ZIKV, four capsid mutations were necessary and sufficient to block viral particle formation (Fig. 3(b), right panel). The YF4 Nano virus was created carrying four capsid mutations in the C25 (Fig. 3(c)). Viral growth characteristics were assayed by IFA post-electroporation (Fig. S1c), replication curve (Fig. 3(d)), luciferase kinetics (Fig. 3(e)), and focus-forming assay (Fig. S1D). These data together show that YF4 Nano, similar to DK23 Nano, was attenuated when compared to YF Nano, but that high titers (8×10^5 FFU/ml) and robust luciferase ($>10^5$ light units) levels could still be attained. YF Nano and YF4 Nano were passaged for ten rounds on Vero cells and their stabilities were evaluated by RT-PCR band size (Fig. 3(f)). The P10 bands were sequenced and P0 and P10 viruses were compared in a luciferase assay. The YF Nano without the four mutations rapidly lost it luciferase expression (Fig. 3(g), top panels), whereas the P10 of YF4 Nano with the designed mutations consistently retained luciferase activities (bottom panels). Together, these results validate that

recombination-dependent lethal mutations are not only effective for stabilizing a reporter ZIKV but can be effectively applied to another flavivirus.

3.4. Reporter virus applications

To establish the utility of these novel reporter viruses, we used both DK23 Nano and YF4 Nano viruses in plaque reduction neutralization tests (PRNT). Reporter viruses have been proposed and used in these assays previously, as a way to increase throughput and decrease assay turnaround time [29,30]. However, due to the bright nature of NanoLuc, we found that these assays can be read as early as 2–4 h post infection (Fig. 2(c) for reporter ZIKV and Fig. 3(e) for reporter YFV, Fig. S2a), which is a significant improvement over the traditional three to five days of a standard PRNT and the 24–48 h of other reporter PRNT assays. The luciferase signals at 2–4 h post infection represent initial translation of input genomic RNA after virus entry [15].

Fig. 4(a) shows a scheme for the reporter neutralization tests. DK23 Nano and YF4 Nano were used to evaluate the neutralizing activity of a panel of sera from mice previously vaccinated/infected with relevant flaviviruses, including ZIKV, YFV, JEV, WNV, and DENV1 to DENV4. Fig 4(b) summarizes the cross-neutralizing titers, whereas Figs. S2b-c present the raw neutralizing curves. When tested against ZIKV DK23 Nano, ZIKV-sera consistently neutralized the virus; only one DENV3-serum weakly neutralized ZIKV; all other flavivirus-sera had NT_{50} values below the initial dilution of 1:50 (Fig. 4(b)). Likewise, when tested against YF4 Nano, only YFV-sera fully neutralized the virus, whereas other sera NT_{50} values were either below or just above the first 1/50 dilution (Fig. 4(b)). Compared with ZIKV, YFV seemed to be weakly cross neutralized by other flavivirus sera. As negative controls, uninfected mouse sera did not neutralize ZIKV though some neutralization was seen with YFV (titer of 1/51). The latter result indicates that the neutralizing titers in the range of 1/50 and 1/64 against YFV should be considered negative (Fig. 4(b)).

Furthermore, a panel of ZIKV-positive human sera were analyzed by both reporter and plaque reduction neutralization tests (Fig. 4(c)) and the results from both were graphed on separate axes for comparison (Fig. 4(d)). The R^2 value of 0.93 affirms that the reporter neutralization results are strikingly similar to those from a traditional neutralization test. Together, these outcomes support the conclusions that (i) reporter viruses could be used for measuring neutralizing antibody titers, (ii) the relative neutralizing levels among different flaviviruses may indicate the type of viral infection, (iii) flavivirus antibodies cross neutralize, (iv) reporter viruses can be used in place of traditional PRNT assays for improved turnaround time.

Finally, we tested the utility of reporter viruses for antiviral testing. Huh7 cells were treated with a known flavivirus inhibitor NITD008 (an adenosine analog [32]) upon infection with the two viruses (Fig. 4(e)). At 48 h post infection, the luciferase signal was inhibited by NITD008 in a dose-responsive manner, leading to EC_{50} of 0.47 μM for ZIKV (Fig. 4(f)) and 0.46 μM for YFV (Fig. 4(g)). These EC_{50} values are equivalent to the previously reported values using plaque reduction assay [31,32], demonstrating the utility of reporter viruses for antiviral testing.

4. Discussion

Reporter flaviviruses, useful tools though they are, have been stymied by instability since they were first published. Efforts to rectify this shortcoming, until recently, have been inadequate. We have developed a method for stabilizing reporter flaviviruses, using recombination-dependent lethal mutations, and shown it to be successful for two flaviviruses, ZIKV and YFV. This strategy relies on two functions that the capsid gene plays in the viral life cycle. The capsid gene codes for the capsid protein, which requires positive charges in
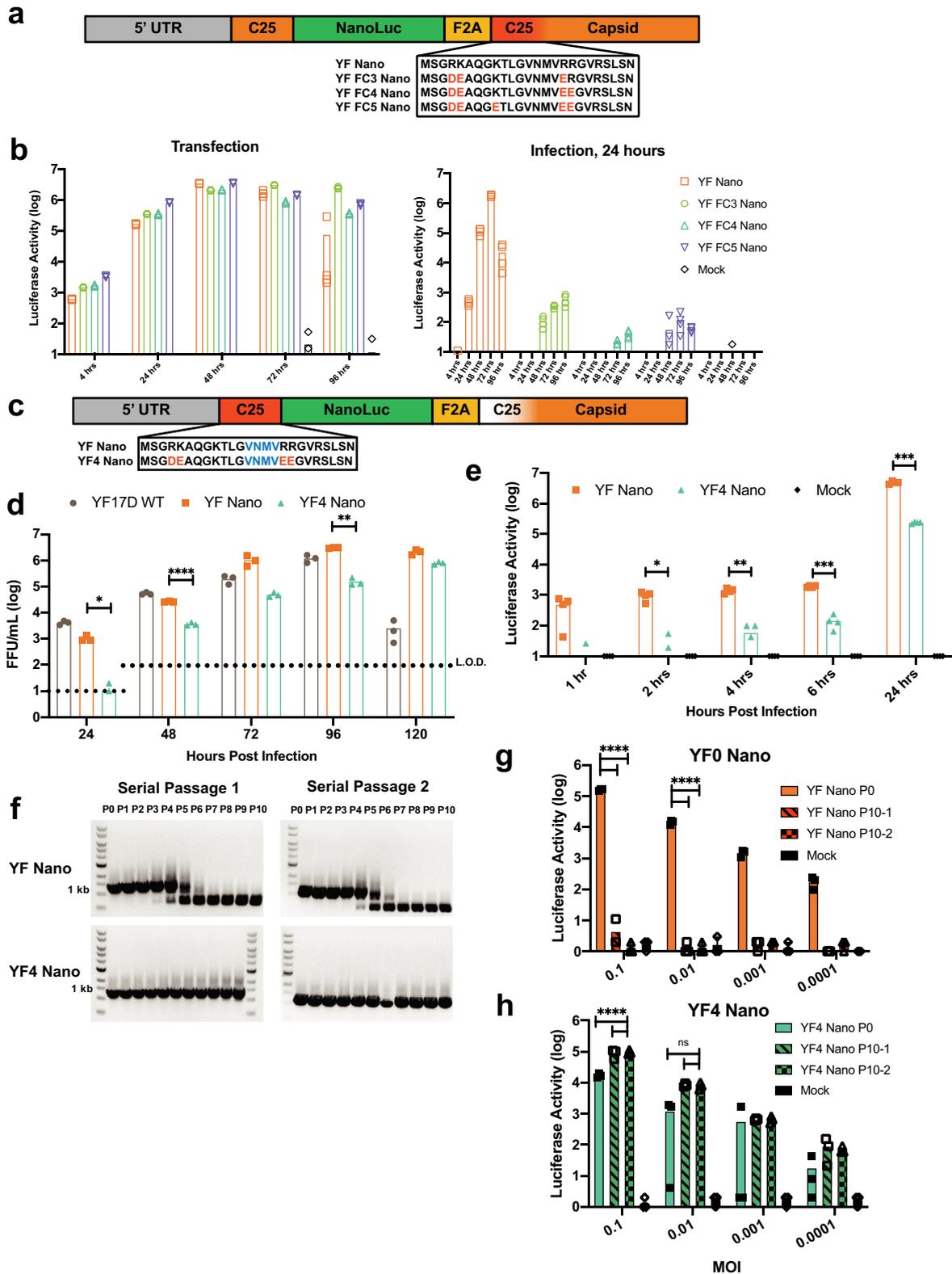
Fig. 3. A stable NanoLuc YFV. (a) Genome scheme of YF17D reporter virus carrying different charge-reversing capsid mutations. These viruses were used to screen deleterious capsid mutations in YFV. (b) YFV capsid mutation screen results. See Fig. 1(d) for experimental scheme. Transfection results for different YFVs with capsid mutations taken at different time points after electroporation show robust luciferase expression. Subsequent infection using infected cell supernatants taken at the indicated time points shows four and five amino acid changes are sufficient for knockdown of viral particle formation. (c) Genome scheme of YF Nano and YF4 Nano, showing positions of capsid mutations (red) and the amino acids that correspond to the 5′ CS (blue). (d) Replication kinetics of YF17D WT, YF Nano, and YF4 Nano on Vero cells (MOI 0.01, $n = 3$). 2-way repeated measures ANOVA with Tukey's post-hoc test was used to assess significance (*=$p < 0.05$, **=$p < 0.01$, ***=$p < 0.001$, ****=$p < 0.0001$). (e) Luciferase kinetics of YF Nano and YF4 Nano on Vero cells (MOI 0.5, $n = 4$). Significant differences in the data were assessed by 2-way ANOVA with Tukey's post-hoc test. (f) Passaging results for YF Nano and YF4 Nano. See Fig 2d for passaging scheme. The RT-PCR band from intact reporter virus is 2388 bp, while the non-reporter band (with luciferase gene deleted) is 631 bp. The consistent band size seen in YF4 Nano indicates its stability through 10 passages. (g) Luciferase assay comparing P0 and P10 luciferase activity between YF Nano and YF4 Nano at different MOIs ($n = 3$). Infection was carried out for 24 h on Vero cells. Significant differences were measured using 2-way ANOVA with Tukey's post-hoc test (*=$p < 0.05$, **=$p < 0.01$, ***=$p < 0.001$, ****=$p < 0.0001$).
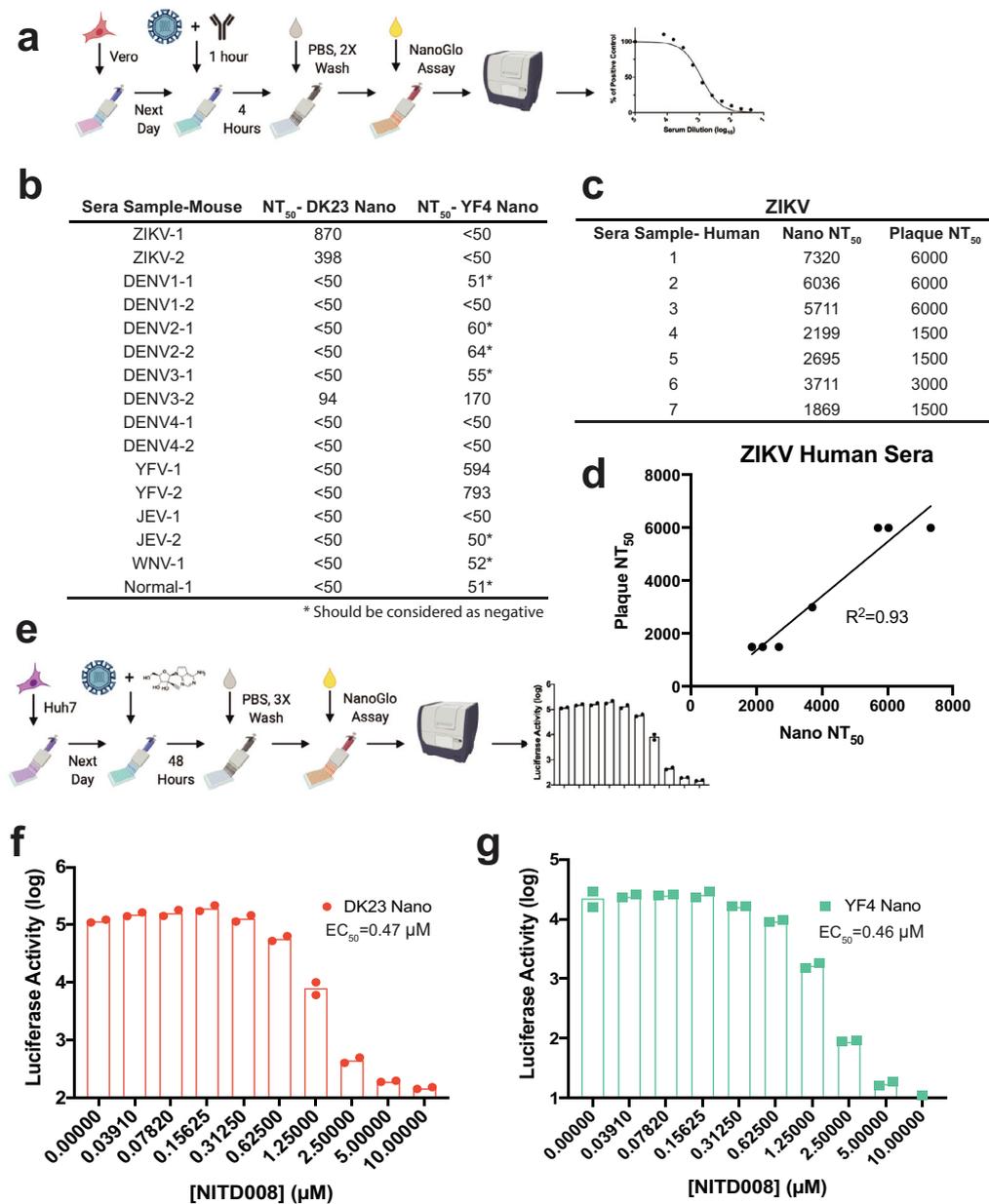
Fig. 4. Applications of stable reporter viruses. (a) Scheme for four-hour reporter neuralization test. Briefly, Vero cells are plated at 1.5×104 cells per well in a 96 well plate. The next day, sera are serially diluted 2-fold (starting at 1:50, $n = 2$) and mixed 1:1 with virus and incubated for 1 h at 37 °C. The sera:virus mixture is plated on the cells and incubated for 4 h. Following PBS wash, the plates are read by plate reader after addition of luciferase substrate. (b) Table with NT50 values of a panel of sera from mice inoculated with the indicated virus. Sera were tested against both DK23 Nano and YF4 Nano viruses. (c) NT50 values from a panel of Zika-positive human sera samples, tested by both 4-h reporter neutralization test and traditional plaque assay. (d) Graphical comparison of reporter and plaque NT50 values. Simple linear regression was used to create a line of best fit. (e) Antiviral assay scheme. Huh7 cells are seeded at 1.5×104 cells per well in a 96 well plate. The following day, reporter virus is mixed with diluted compound and plated on the cells ($n = 2$). Following a 48-h incubation, cells are washed, and luciferase substrate is added. Luciferase levels are read by plate reader. (f,g) Antiviral activity against DK23 Nano and YF4 Nano, respectively, assessed by luciferase levels.

the N-terminus, though their role is still unclear. It has been proposed that they assist in binding viral RNA [25], though models of capsid-RNA binding have been suggested that do not include the N-terminus [33,34]. The structure of the N-terminus is flexible and unstructured and accordingly this region has yet to be incorporated in solved crystal structures. Though its function remains obscure, our results show that multiple flaviviruses, not just DENV, require a threshold of positive charges in this region for virion assembly.

The capsid gene also provides RNA replication elements essential for viral replication. The flavivirus 5′ UTR is highly structured, with two well defined stem loops, A and B. The start codon (the beginning of the capsid gene) is present on the descending side of stem loop B. Other required RNA signals in the capsid gene include the well-defined 5′CS [35], the cHP [36], 5′ DAR [37], and DCS-PK [38]. Ideally, the engineered capsid mutations that stabilize the reporter flaviviruses would change the charge, but not perturb these cis-acting elements. The attenuation of replication seen when comparing DK Nano and DK23 Nano or YF Nano and YF4 Nano indicates we have not wholly achieved this ideal condition. Exhaustive screening of mutations in this region may yield a mutant that is more replication robust compared to what has been published here, though it is unlikely to be completely unattenuated. It is possible that simply increasing the length of the genome perturbs genome cyclization or viral RNA packaging and thus agitates the viral life cycle. As demonstrated, the attenuation inherited with the stability does not inhibit the virus' capacity for virulence, nor its utility as a screening and

serological tool. We have shown this system is valuable for rapidly testing sera for neutralizing antibodies and compounds for antiviral activity, though the number of sera samples and compounds tested was relatively small. Further validation with more sera samples, including those with a broad range of neutralizing activity, and antiviral compounds is warranted.

Recently, Volkova et al. published a stable Zika NanoLuc and EGFP virus. Their strategy, analogous to ours in that it stops viruses that have recombined, involves adding a frameshift mutation at the beginning of the duplicated capsid. This elegant approach involves very little perturbation of RNA elements and still results in a stable virus [24]. It should be noted that the C25 NanoLuc ZIKV here reported is significantly more robust than their described C25 ZIKV, as seen by comparison of viral titers from growth kinetics on Vero cells. Another elegant and successful approach in flavivirus reporter constructs was first reported in [39] and further improved on in [40]. A split NanoLuc construct was utilized and engineered in a permissive site in JEV NS1. Such a small insertion (11 amino acids) lends itself to great stability and Tamura et al. modeled and tested insertion sites until the reporter virus and WT JEV had no statistical difference in mortality in mice. Despite stability and lack of attenuation, this system sacrifices the flexibility present when the virus codes the full protein, most notably being unable to perform *in vivo* imaging.

The long-term stability of reporter flaviviruses could be used to further probe the complicated, multi-host life cycle of these human pathogens. *In vivo* imaging of ZIKV transmission between live mice and mosquitoes was the original goal of this research project. Unfortunately, neither the DK Nano nor the DK23 Nano virus succeeded in causing disease in A129 mice or showing robust luciferase signal in an IVIS setup. Rectifying these shortcomings are ongoing research aims.

In conclusion, we have achieved a stable NanoLuc ZIKV and YFV by incorporating recombination-dependent lethal mutations that abolish formation of viral particles if they have recombined. These viruses have potential to be used in enhanced, rapid-turnaround, PRNT assays and drug screenings. They can also be used as a platform for stable, long-term transgene delivery.

Declaration Competing of Interest

Dr. Shi and Dr. Xie have a patent Reverse genetic system of Zika virus (Patent ID: US 10,533,997) issued. The authors report no other conflicts of interest.

Data availability

Full length sequences of DK Nano, DK23 Nano, YF Nano, and YF4 Nano can be found on Genbank, accession numbers MT505349, MT505350, MT505351, and MT505352, respectively.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.102838.

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