A comprehensive influenza reporter virus panel for high-throughput deep profiling of neutralizing antibodies

Broadly neutralizing antibodies (bnAbs) have been developed as potential countermeasures for seasonal and pandemic influenza. Deep characterization of these bnAbs and polyclonal sera provides pivotal understanding for influenza immunity and informs effective vaccine design. However, conventional virus neutralization assays require high-containment laboratories and are difficult to standardize and roboticize. Here, we build a panel of engineered influenza viruses carrying a reporter gene to replace an essential viral gene, and develop an assay using the panel for in-depth profiling of neutralizing antibodies. Replication of these viruses is restricted to cells expressing the missing viral gene, allowing it to be manipulated in a biosafety level 2 environment. We generate the neutralization profile of 24 bnAbs using a 55-virus panel encompassing the near-complete diversity of human H1N1 and H3N2, as well as pandemic subtype viruses. Our system offers in-depth profiling of influenza immunity, including the antibodies against the hemagglutinin stem, a major target of universal influenza vaccines.
Influenza virus continues to cause seasonal epidemics and pandemics despite vaccine availability. In addition, highly pathogenic avian influenza viruses cause sporadic outbreaks in humans with a high mortality rate, posing potential risk of human adaptation and are considered pandemic threats. Viral glycoprotein hemagglutinin (HA) is required for viral entry into the host cell through binding to sialic acid moieties on glyco-proteins or glycolipids and mediates fusion of the viral membrane with host endosomal membranes. Another viral glycoprotein neuraminidase (NA) cleaves sialic acid and promotes the release of progeny viruses from infected cells. Neutralizing antibodies are primarily directed against HA and can compete for receptor binding, inhibit the membrane fusion machinery, or cause virus aggregation. Antibodies to NA can sometimes have neutralizing activity but are thought to primarily limit virus egress by inhibiting enzymatic activity and release of virus thereby inhibiting viral spread. Based on the antigenic properties of the HA and NA, influenza A viruses are divided into groups 1 and 2, each of which have several subtypes defined primarily by HA. To date, there are 18 HA and 11 NA subtypes identified and characterized. Currently, strains of H1N1 and H3N2 influenza A as well as influenza B viruses co-circulate in humans. In addition, several other subtypes of animal influenza viruses (e.g., H5N1, H6N1, H7N9, H9N2, and H10N8) can also infect humans and occasionally result in mortality. In contrast to influenza A viruses, which have an extensive zoonotic reservoir, influenza B viruses are isolated almost exclusively from humans with a more limited evolutionary history and have diverged into only two genetically and antigenically distinct lineages (Victoria- and Yamagata-like lineages).

The discovery of broadly neutralizing antibodies (bnAbs) capable of neutralizing multiple influenza virus subtypes in humans opens an opportunity for developing a universal influenza vaccine, which elicits such antibodies. Many of these antibodies target conserved epitopes in the HA stem and neutralize virus by inhibiting the viral fusion machinery, therefore, the activity is not detectable by traditional hemagglutination inhibition (HAI) assay, which measures the ability of antibody to inhibit virus–receptor interaction. Several less broad bnAbs recognize the receptor-binding site (RBS) in the HA head, hence exhibiting HAI activity. Since most universal influenza vaccine candidates aim to induce protective levels of such bnAb response, comprehensive analysis of the neutralization breadth and potency regardless of HAI activity is crucial to accelerate the efforts to develop effective universal influenza vaccines. The influenza microneutralization (MN) assay has been the most commonly used method to measure neutralizing activity of antibodies against influenza virus. In this assay, virus replication is measured by either detecting viral nucleoprotein (NP) with an enzyme-linked immunosorbent assay (ELISA), titrating hemagglutination, or scoring cytopathic effects. Plaque-reduction neutralization assay is also commonly used for influenza and other viruses. These approaches are labor-intensive, not easily scalable, and the handling of live viruses of animal origin requires high-containment laboratories. There is also significant performance variability between laboratories due to multi-step signal amplification or reliance on subjective scoring. Given these inevitable limitations of the current MN assay, there is a need to transform the MN assay to be safe, high throughput, robust, easy to standardize, and automation compatible.

The use of reporter viruses for developing standardized high-throughput neutralization assays has greatly expanded our ability to measure and characterize antibody responses induced by infection and/or vaccination. While HA/NA pseudotyped reporter lentiviruses have been constructed for influenza and utilized effectively for measuring neutralizing activity, they are often criticized for not recapitulating some of the key aspects of influenza viruses, such as viral morphology and HA spike density. Therefore, building influenza viruses with a reporter feature is an attractive alternative. Among several approaches to produce influenza reporter viruses, a replication-competent reporter virus can be developed by fusing a reporter gene to a viral gene (e.g., PA or NS). This new virus remains as virulent as the parental virus in animals, making it suitable for viral pathogenesis studies, yet still subject to biosafety precautions. In contrast, single-cycle infectious or replication-restricted reporter (R3) influenza viruses can be generated by replacing one of the viral essential genes (e.g., PB1 or HA) with a reporter gene. Thereby these R3 viruses are capable of replicating only in cells complementing the deleted viral gene product in trans, making it safe to handle in low-containment laboratories.

In the present study, we developed a comprehensive panel of R3 viruses to enable high-throughput and in-depth influenza virus neutralization profiling. We generated a neutralization matrix of 24 monoclonal antibodies (mAbs) and 53 R3 viruses spanning 5 subtypes of influenza virus and compared the R3 virus-based assay with the gold standard ELISA-based MN assay. The reporter virus assay provides a more robust method for probing the breadth of anti-influenza immunity needed for developing universal influenza vaccines.

**Results**

**Generation of R3APB1 viruses.** The influenza virus has a segmented genome, which allows its rapid and relatively easy genetic manipulation. Each segment consists of either one or two protein-coding sequences flanked by short noncoding regions (NCRs). The genome packaging signal sequences are located at both 3′- and 5′-termini of each segment encompassing the entire NCRs and part of the adjacent protein-coding sequence. Genome packaging sequences are unique to each segment and required for an efficient incorporation of each viral RNA molecule into the virion. The PB1 segment encodes two proteins: PB1, the main component of the viral RNA-dependent RNA polymerase, and PB1-F2, translated from the +2 frame and not required for virus replication. The protein-coding sequence of PB1 (A/WSN/1933, H1N1) has 2,274 nucleotides and is flanked by 24 bases at the 3′- and 43 bases at 5′-termini. In addition to the NCRs, genome packaging signals of the PB1 segment comprise 120 bases of the coding region at both 3′- and 5′-termini (Supplementary Fig. 1).

To build R3 influenza viruses, we altered the PB1 segment by removing the PB1 coding sequence not required for genome packaging and replacing it with the reporter encoding fluorescent protein tdKatushka. To prevent translation of alternative transcripts from PB1 segments, we mutated potential initiation codons (ΔATGs) found between the 3′ end and the reporter open reading frame (ORF) (Fig. 1a). PB1 is essential for virus replication therefore, R3 influenza viruses in which PB1 was removed (R3ΔPB1) can be propagated only in cells expressing the PB1 in trans (Fig. 1b, c). Thus, we used MDCK-SIAT1 cells, which constitutively express human β-galactoside α2,6-sialyltransferase 1 (SIAT1) to prepare a cell line constitutively expressing PB1 of A/WSN/1933 by transfecting a plasmid encoding a puromycin-resistance gene, PB1, and a self-cleaving peptide derived from *Thoesa asigna* virus 2A (T2A) in between the two genes. The R3ΔPB1 virus was rescued by reverse genetics using 8 plasmids encoding HA and NA of wild-type H1N1 or H3N2 influenza A viruses; PB2, PA, NP, M, and NS of A/WSN/1933; and another encoding the reporter. To validate the use of R3ΔPB1 viruses for influenza neutralization assays, we also rescued replication-competent H1N1 and H3N2 parental viruses,
expression (Supplementary Fig. 2). These results demonstrate that replicates similarly in MDCK-SIAT1 cells with or without PB1 replicates only in PB1-expressing cells, whereas parental virus infected cells were detected either by ELISA with anti-NP antibody or by fluorescence using an automated image-based plate reader at 24 h post infection. We measured neutralizing activity (80% inhibitory concentration, IC_{80}) detected by both ELISA- and fluorescent reporter-based readouts for several mAbs against a total of 8 R3ΔPB1 viruses (4 H1N1 and 4 H3N2 viruses) and found a strong positive correlation (Pearson r = 0.94, p < 0.001) between the two IC_{80} datasets, demonstrating that the fluorescent reporter-based readout yields comparable neutralization results to conventional ELISA-based detection method (Fig. 2a and Supplementary Fig. 3).

To assess the R3ΔPB1 viruses with the MN assay format, we next determined neutralizing IC_{80} for 6 mAbs against matched pairs of R3ΔPB1 and parental viruses (4 H1N1 and 3 H3N2 viruses). Cells infected with R3ΔPB1 viruses were detected by fluorescence, while cells infected with parental viruses were detected by ELISA. The positive correlation (Pearson r = 0.87, p < 0.001) between neutralization IC_{80} of mAbs against R3ΔPB1 and parental viruses shows that R3ΔPB1 viruses retained the neutralization sensitivity of parental viruses with matched HA and NA.

Building a comprehensive panel of R3 viruses. We aimed to build a comprehensive panel of R3ΔPB1 viruses spanning the entire antigenic evolution of human H1N1 and H3N2 subtype viruses. We selected representative influenza strains based on phylogenetic analysis of HA sequences deposited in public databases, literatures on genetic and antigenic evolution of human H1N1 and H3N2 influenza viruses, and vaccine strains utilized since 1930^{22,23,25-28}.

H1N1 subtype virus was introduced into the human population in 1918 and circulated until it was replaced by H2N2 virus in 1957. H1N1 virus re-emerged in 1977 and circulated until 2009. During this period, H1N1 viruses evolved significantly through genetic drift into multiple clades with distinct genetic and antigenic properties^{27,29-31}. To capture the antigenic variations of these viruses, we chose 7 matched HA and NA sequences from viruses circulating between 1933 and 1957 and 13 HA and NA sequences from viruses circulating between 1977 and 2009. The 2009 pandemic H1N1 virus acquired sustained human-to-human transmission and rapidly and completely replaced the pre-pandemic H1N1 virus. Since its emergence, the 2009 pandemic H1N1 virus has accumulated several amino acid substitutions linked to changes in antigenicity^{32}. Therefore, we included 3 HA and NA sequences from viruses isolated between 2009 and 2015 and HA and NA sequences of swine-origin A/New Jersey/8/1976 (H1N1), which caused an isolated outbreak in 1976 in the United States. In summary, our H1N1 panel consists of 19 pre-pandemic strains, 3 pandemic strains, and 1 swine-origin H1N1 strain (Fig. 3a). Of note, our panel of H1N1 viruses includes genetically similar viruses to all World Health Organization (WHO)-recommended H1N1 vaccine

which possess HA and NA of wild-type viruses and the internal genes including PB1 of A/WSN/1933, and propagated in MDCK-SIAT1 cells (Fig. 1b, c).

To examine the morphology of R3ΔPB1 virus (A/Michigan/45/2015, H1N1), we performed negative stain electron microscopy using reverse genetics of eight plasmid system with PB1 and the corresponding wild-type segment.

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spg?decorator=influenza), antigenically representative viruses circulating in humans in the 1940s, or representatives of the major HA and NA lineages of seasonal H1N1 viruses.

H3N2 subtype virus has been circulating in humans since its emergence in 1968. Comprehensive genetic and antigenic analysis of human H3N2 viruses groups them into 14 distinct antigenic clusters, and hence, we include several representatives from each antigenic cluster in our panel. Sporadic outbreaks with swine H3N2 variant (H3N2v) have also been described in humans. Therefore, we included the swine-origin H3N2v strain, A/Indiana/10/2011. As a result, our H3N2 panel includes 25 human H3N2 strains and 1 H3N2v strain (Fig. 3b).

When we calculate the conservation of solvent-exposed surface among H1 and H3 HAIs included in our panel, we notice that the head region of HA of both H1 and H3 is substantially more variable while the stem region is mostly conserved as expected (Fig. 3c, d). The degree of surface conservation reflects that of much larger datasets of H1 and H3 HAIs (Fig. 3c, d), suggesting that the selected HA sequences in our R3 virus panel capture the HA diversity of human H1N1 and H3N2 viruses.

Generation of R3ΔHA viruses with highly pathogenic influenza virus sequences. Working with highly pathogenic influenza viruses (e.g., H5N1, H7N9, 1918 H1N1) or influenza lineages disappeared from human population (e.g., H2N2) requires high containment laboratories. Although R3ΔPB1 viruses have limited capacity to replicate due to the requirement of PB1 complementation, they retain the ability to reassort the HA segment with wild-type influenza viruses. To prevent this event, we evaluated alternative approaches less susceptible to reassortment and generated reporter viruses expressing HA and NA of viruses with pandemic potential.

First, we developed influenza viruses, unable to reassort HA segments, by making "rewired" R3ΔPB1 viruses (R4ΔPB1) as previously described. For this purpose, two segments of influenza genome were altered: the PB1 segment was modified to encode HA,
and the HA segment was modified to encode the tdKatuhka2 reporter. Using these altered PB1 and HA segments and reverse genetics, we were able to rescue R4ΔPB1 A/Switzerland/9715293/2013 (H3N2) virus in PB1-expressing cells (Supplementary Fig. 4a–c).

By measuring neutralizing IC80 of 15 mAbs for both R3ΔPB1 and R4ΔPB1, we found a strong positive correlation (Pearson r = 0.94, p < 0.001) between IC80 neutralizing activity against the two viruses, demonstrating that these viruses have equivalent neutralization sensitivities (Supplementary Fig. 4d). Moreover, we found that the R4ΔPB1 A/Switzerland/9715293/2013 (H3N2) virus did not reassort its HA segment with A/Solomon Islands/03/2006 (H1N1) when the two viruses were co-infected and propagated on PB1-expressing cells (Supplementary Fig. 4e). Although these results show that R4ΔPB1 viruses can be safely rescued and used for high-throughput neutralization assay, further work is required to optimize the rescue and the propagation of R4ΔPB1 to be a practical and deployable process for producing reporter viruses.

Next, we explored an alternative approach to rescue R3 viruses unable to reassort the HA segment by replacing the HA coding sequence with the reporter gene (R3ΔHA). In this configuration, the virus lacks a functional HA segment and is unable to reassort the segment with other viruses (Fig. 4a–c), and it will be able to replicate only in HA-expressing cells19. To assess R3ΔHA viruses for the reporter neutralization assay, we generated R3ΔHA A/Switzerland/9715293/2013 (H3N2) virus to compare the neutralization IC80 of several mAbs against both R3ΔPB1 and R3ΔHA viruses. There was a positive correlation between neutralization IC80 determined with these viruses (Pearson r = 0.90, p < 0.001), although we noticed slight differences in sensitivity to neutralization when testing anti-stem antibodies against R3ΔPB1 and R3ΔHA viruses (Fig. 4d). Using this approach, we prepared 6 R3ΔHA viruses (i.e., 2 H5N1 of clade 1, A/Vietnam/1203/2004, and clade 2.1.3, A/Indonesia/05/2005, 2 genetically and antigenically related H7N9, A/Anhui/01/2013 and A/Shanghai/02/2013, 1 H2N2, A/Singapore/1/1957, and 1 H10N8, A/Jiangxi-Donghu/346-2/2013) in addition to the 49 R3ΔPB1 virus panel36,37.
Characterizing influenza responses in serum samples from human vaccinees using R3 viruses. To assess neutralization activity in polyclonal sera using R3 viruses, we measured the neutralization titers in samples collected in the Phase I human clinical trials of experimental H5N1 and H7N9 vaccines conducted by the Vaccine Research Center in 2011 and 2015, respectively (NCT01086657 and NCT02206464, respectively). Briefly, healthy adult volunteers were immunized with DNA encoding H5 (A/Indonesia/05/2005) or H5N1 monovalent influenza vaccine (MIV), or DNA encoding H7 (A/Shaanghai/02/2013) HA, H7N9 MIV, or combinations of H7 DNA and H7N9 MIV followed by a boost with H5N1 MIV or H7N9 MIV, respectively. Neutralization titers were measured at the time of vaccination (week 0) and 2 weeks post-boost against vaccine-matched strain (i.e., H5N1 A/Indonesia/05/2005 or H5N1 monovalent influenza vaccine (MIV), or DNA encoding H7 (A/Shaanghai/02/2013) HA, H7N9 MIV, or combinations of H7 DNA and H7N9 MIV followed by a boost with H5N1 MIV or H7N9 MIV, respectively). Neutralization titers were measured at the time of vaccination (week 0) and 2 weeks post-boost against vaccine-matched strain (i.e., H5N1 A/Indonesia/05/2005 or H5N1 monovalent influenza vaccine (MIV), or DNA encoding H7 (A/Shaanghai/02/2013) HA, H7N9 MIV, or combinations of H7 DNA and H7N9 MIV followed by a boost with H5N1 MIV or H7N9 MIV, respectively). 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These results suggest that our fluorescent plaque-reduction assay with influenza R3 viruses allows live, high-throughput measurement of viral inhibitory activities targeting NA and will facilitate the characterization of novel vaccine candidates and mAbs with anti-NA responses.

Profi ling of influenza neutralizing mAbs with a 55-virus panel. Using our comprehensive panel of R3 influenza viruses spanning 6 different influenza A subtypes, we profiled the neutralization activities of serum antibodies from human vaccinees using R3 viruses. This panel included 11 H1N1, 16 H2N2, 15 H3N2, 4 H4N1, 4 H5N1, and 3 H7N9 viruses. The neutralization assays were performed at least twice and representative data are plotted.
breadth and potency of a total of 24 human mAbs. Thirteen of them were isolated at the Vaccine Research Center from peripheral blood mononuclear cells collected as part of the H5N1 or H7N9 vaccine clinical trials39,43–45, while 11 other antibodies were previously described elsewhere46–55. Among 24 antibodies, 18 antibodies recognize epitopes on the conserved HA stem region and 6 antibodies (i.e., CH65, 5J8, C05, F005-092, F005-126, and 310-33-1F04) bind within the HA head region. HA stem-binding 315-53-1A07 which did not neutralize any of the viruses was included in our panel. Of note, neutralization IC₈₀ values of the mAbs did not change drastically between 18 and 24 h after infection while the virus-infected cells dramatically increased during this time period (Supplementary Fig. 7), providing a reasonable flexibility for assay operation and fluorescent readout. We generated a matrix of neutralizing profiles for 24 antibodies against 55 R3 viruses consisting of 1320 data points and analyzed the matrix by hierarchical clustering (Fig. 5).

Despite the fact that there is no associated information about viruses in the input dataset, the profile matrix segregates not only groups of viruses but also virus subtypes into defined clusters (Fig. 5). Interestingly, the hierarchical clustering groups several antibodies according to their immunogenetic composition or convergent antibody class44,56,57. For example, MEDI8852 is clustered with 2 other antibodies (315-53-1F12 and 315-53-1B06) and all the three antibodies belong to the V₅₆₁-6₁ + D₁₂₃-3 convergent multi-donor class57, while CR9114 is clustered with CR6261 and 315-02-1H01, which all shares V₅₆₁-6₁. The latter case is particularly noteworthy as CR9114 has much broader neutralizing capacity than the other two, yet clustered with the stereotypical group 1-specific V₁₁-6₉ antibodies CR6261 and 315-02-1H01 (Fig. 5).

Deep characterization of neutralizing profiles also predicts the developmental pathway for each bnAb. The convergent multi-donor bnAbs in V₁₈ QXXV class (i.e., 315-09-1B12 and CT149) neutralize many group 2 viruses while having very limited breadth against group 1 viruses (Fig. 5). This confirms previous findings in which the unmutated common ancestor (UCA) of this class of bnAbs engages only group 2 HAs and acquires group 1 reactivity through somatic hypermutation57. Conversely, the V₅₆₁-6₁ + D₁₂₃-3 class bnAbs (i.e., MEDI8852, 315-53-1F12, and 315-53-1B06) possess higher neutralization potency against group 1 viruses than group 2 viruses (Fig. 5), and this is consistent with the preferential engagement of group 1 HAs to the UCAs of this class57. This deep neutralization profiling dataset also allows us to generate neutralization breadth–potency curves at relatively high resolution (Fig. 6). Previous studies used a dataset generated by pseudotyped lentiviral neutralization assays with 15–17 selected HA–NA

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**Fig. 5** In-depth neutralization profiling of influenza monoclonal antibodies. Heatmap of neutralization titers (IC₈₀ μg ml⁻¹) of 24 monoclonal antibodies against a panel of 55 R3 influenza A viruses. Virus subtypes are color-coded and indicated at the bottom of the heatmap. Open and closed circles indicate specificity of monoclonal antibodies and are shown on the right of the heatmap (open, HA head-directed; closed, HA stem-directed). Convergent classes of bnAbs with specific gene signatures are indicated on the left of the heatmap as triangles (gray, V₅₆₁-6₁; black, V₅₆₁-6₁ + D₁₂₃-3; open, V₅₆₁-18 QxxV). Heatmap and unsupervised clustering was made with the ClustVis server (https://biit.cs.ut.ee/clustvis/). Note: H1N1 and H3N2 viruses are R3ΔPB1 and all other viral subtypes are R3ΔHA viruses. Neutralization assays were performed at least twice and representative data are plotted.
sequences45,57 and provided a limited understanding of neutralization breadth both because of the relatively small number of strains and the hypersensitivity of the pseudotyped lentivirus assay format. By determining the neutralization profiles using our comprehensive R3 virus panel, we found two antibodies (MEDI8852 and Fl6v3) that were capable of neutralizing all 55 viruses (Fig. 6). The other two V_{H}6-1 + D_{G}3-3 class mAbs, 315–53-1F12 and 315–53-1B06, neutralized 52 (94.5%) and 54 (98.2%) of 55 viruses, respectively. Although CR9114 neutralized 48 out of 55 viruses (87.3%) its breadth–potency plot demonstrated a biphasic curve, highlighting the preferential neutralization of group 1 viruses (the first phase) and lower potency against group 2 viruses (the second phase) by this antibody (Fig. 6). The RBS-directed antibody C05 showed limited neutralization breadth (32.7%) yet was extremely potent (Fig. 6).

Overall, the neutralization profiling performed with the reporter influenza virus assay provides less biased breadth and potency information than the pseudotyped lentivirus assay or highly variable traditional MN assay. High-resolution information may also help predict the class and the origin of antibodies with particular neutralization signatures.

**Discussion**

Current influenza vaccines are imperfect and there is a large room to improve their efficacy, consistency, and breadth. Historically, efficacy has been associated with serum HAI activity58,59 and the HAI assay was developed as a surrogate for virus neutralization60,61. To improve current vaccines and to develop new vaccine concepts that will have broader and more durable efficacy, accurate measurement of neutralizing activity across a broad range of influenza viruses is critical. The HAI assay is a surrogate only for neutralization directed at the RBS on HA, therefore neutralization targeting the HA stem or other relatively conserved sites on HA and NA will not correlate with HAI. In addition, some new drifted seasonal strains of influenza and subtypes with pandemic potential do not always have predictable hemagglutination properties. Therefore, to encompass the variety of HA and NA strains and the antibodies induced by diverse vaccine approaches, emphasis on a reproducible and authentic measurement of virus-neutralizing activity is needed.

Unlike the HAI assay that measures the antibody’s ability to block receptor binding of viral HA, plaque reduction62 and MN63 assays can capture neutralizing antibodies that block any virus replication step, including attachment, internalization, pH-induced conformational change of HA, membrane fusion, and virus egress. Despite efforts initiated by WHO, execution of influenza neutralization assays is still not standardized, leading to significant variability among results reported by different laboratories9,63. Development of a large panel of representative influenza viruses and significant improvement of assay throughput and safety are highly anticipated in the field and will enable deep characterization and systematic comparisons of influenza virus-neutralizing antibody responses. The virus panel combined with serum and mAb standards will allow normalization across assay platforms and facilitate the evaluation of universal influenza vaccine candidates. In the present study, we address these outstanding needs by building an engineered influenza reporter virus-based neutralization assay coupled with high-throughput image-based readout in a biosafety level 2 setting. Our panel consists of 55 reporter viruses capturing almost the entire antigenic evolution of human H1N1 and H3N2 subtypes as well as historical human H2N2 and three other subtypes circulating at the human–animal interface. Moreover, we were able to rescue and propagate R3 chimeric influenza viruses encoding the HA and NA of influenza A using the internal genes of A/WSN/1933 influenza A virus. R3ΔPB1 chimeric influenza A/B viruses can replicate only in cells expressing influenza A PB1 in trans. This approach to rescue viruses expressing HA and NA of influenza B viruses was described in several previous reports64–66, and it was shown that chimeric influenza A/B viruses have similar growth profiles as wild-type influenza B viruses66. Although none of the 24 antibodies included in our study neutralized these R3ΔPB1 chimeric influenza A/B viruses, we have successfully utilized these
viruses in neutralization assays to assess anti-influenza B virus-neutralizing antibody responses elicited by experimental vaccines.67

Direct detection of fluorescent reporter allowed us to measure virus replication in live cells without additional signal amplifications unlike ELISA- or hemagglutination-based readout. Image-based readout was chosen to further facilitate fast and precise data acquisition and analysis. In contrast to ELISA-based assays, in which the signal is measured for the entire well after a series of signal amplification steps, image-based detection provides higher signal-to-noise ratio, dynamic range, and precision at single-cell resolution. The R3 virus neutralization assays can be readily automated to minimize hands-on time and human errors. It is also worth noting that the fluorescent image-based readout makes the assay more cost-effective compared with other assays such as ELISA- and luciferase-based assays. By using R3 viruses, we can detect virus-inhibitory activity of both anti-HA and anti-NA antibodies by counting number of fluorescent cells or measuring size of fluorescent plaques in a safe and high-throughput manner.

Recent attempted improvements of the WHO-recommended ELISA-based MN assay protocol88–70 underscore the need for the development of more reliable assays. As noted, previous reports have described the potential utility of R3APB1 and R3ΔHA19,20 influenza viruses in neutralization assays. However, these efforts did not lead to widespread adoption and the development of standardized influenza neutralization assays due to the small number of available reporter viruses, impediments to access essential reagents (e.g., influenza reverse genetics plasmids and stable cell lines), and lack of comprehensive validation. We found that the antigenicity and neutralization sensitivity of R3APB1 is similar to that of authentic influenza viruses, while R3ΔHA viruses appeared to be slightly more sensitive to neutralization than R3APB1. This is likely due to less efficient HA incorporation into R3ΔHA viruses19,20 and represents a common feature of R3ΔHA viruses. Although we noticed that the neutralization sensitivity of R4ΔPB1 closely matched that of R3ΔAPB1 implying their potential superiority for building viruses that are unlikely to reassort, additional optimization of R4ΔPB1 virus is necessary to become a practical alternative due to its poor rescue efficiency. Although the use of R3 (and R4) viruses offers further advancements in the assay throughput, safety, and precision, this approach has its own limitations. Since the reporter segment is not required for viral replication, it is inevitable that after extensive virus passage, the reporter segment may accumulate mutations made by the error-prone viral RNA polymerase, which in turn, will diminish reporter gene activity and/or expression. However, it is possible to prepare virus stocks relatively quickly with five or less passages, and we confirm that these stocks retain an active reporter gene expression. Additionally, genomic stability of R3 influenza viruses can be improved by utilizing the variant PB1 polymerase with lower error rates as reported recently71 and designing R3 viruses capable of replicating only when the engineered genomic segment is functional, such as inducible gene-expression systems.

As we continue to expand our collection of viruses and neutralizing antibodies, we will be able to perform neutralization fingerprint analysis72 to provide a better understanding of the relationships between fine epitope specificity and neutralization breadth and potency and allow computational predictions of the epitope-specific contributions of polyclonal serum antibodies to overall neutralizing activity. This type of analysis will foster the immune monitoring of antibody responses elicited by universal influenza vaccine candidates, particularly those targeting the conserved HA stem supersite73–75. By describing our methods and depositing sequences required to generate R3 viruses, we provide the basis for implementing this technology for the strains reported here as well as for future emerging viruses. We anticipate that our assay will foster rapid discovery and characterization of influenza bnAbs as well as virus-inhibitory antibodies and facilitate efficient evaluation of vaccine-elicited antibody responses for accelerating the efforts to develop universal influenza vaccines.

Methods

Plasmids. To prepare influenza reverse genetics plasmids for rescue of influenza A H1N1 or H3N2 viruses described in this study, HA and NA coding sequences were retrieved from Genbank; NCRs of A/WSN/1933 for H1N1 or A/Netherlands/009/2010 for H3N2 viruses were added at both ends. Full-length HA and NA sequences were cloned into a dual promoter influenza reverse genetics plasmid previously described76. To rescue influenza viruses, dual promoter plasmids encoding internal genes of A/WSN/1933 (pHW181-PB2, pHW182-PB1, pHW183-PA, pHW185-NP, pHW187-M, pHW188-NS) were used.77 To prepare PB1 reporter segment used to rescue R3 viruses, the sequence containing the PB1 genome packaging signals of A/WSN/193324 and mKate2 or tdKatushka2 reporter coding region23 (Addgene Cat No. 56049) was synthesized and cloned using BsmBI (New England Biolabs) restriction sites into the dual promoter influenza reverse genetics plasmid. Similarly, HA reporter segment was synthesized with HA genome packaging signals of A/Puerto Rico/8/193477 flanking the tdTakashika2 reporter sequence and cloned into the dual promoter influenza reverse genetics plasmid using BsmBI restriction sites. To prepare the influenza reverse genetics plasmid using chicken beta-actin CAG pol-II promoter (Addgene Cat No. 41583), an insert comprising human pol-I promoter and mouse pol-I terminator sequences in negative orientation flanking dual BsmBI restriction sites was synthesized using KpnI and XhoI restriction sites. Then full-length of influenza genes of high-yield A/Puerto Rico/8/193477 were cloned into BsmBI restriction sites. To prepare plasmids for stable cell line development, sequences of Streptomyces pyrourycin N-acetyltransferase (PAC), which confers resistance to pyrourycin, followed by self-cleaving peptide of Thoscis asigna virus RTase (and coding region of influenza A PB1, which has PB1-E2 transcript in unmodified, or HA genes were synthesized and cloned into pCAGGS plasmid (Addgene Cat No. 41583) using KpnI and Xhol restriction sites. All plasmids were confirmed by Sanger sequencing.

Cells. To propagate influenza viruses, MDCK-SIA1T1 cells (Millipore Sigma) were used. Cells were maintained with complete media comprising Dulbecco’s modified Eagle’s medium high glucose (DMEM; Thermofisher) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gemini Bio-Products), 100 units ml−1 penicillin (Thermofisher), 100 μg ml−1 streptomycin (Thermofisher), and geneticin (1 mg ml−1) (Thermofisher). To develop constitutively PB1- or HA-expressing MDCK-SIA1T1 cells, one plasmid encoding both pyruvokin resistance and influenza genes was transfected into MDCK-SIA1T1 cells using Lipofectamine 2000 (Thermofisher). Two days post transfection, cells were transferred from 6-well plates into 10-cm dishes using DMEM media with 10% FBS. Whole-cell lysates were collected from MDCK-SIA1T1 cells (positive orientation) and pol-I (negative orientation) promoters was used to rescue influenza viruses, dual promoter plasmids encoding internal genes of A/WSN/1933 obtained from St. Jude Children’s Research Hospital and a CMV-driven plasmid expressing internal genes of A/Puerto Rico/8/193477 were amplified using the Qiagen One-step RT-PCR Kit (Qiagen). Influenza viral RNA was amplified using the Quagen One-Step RT-PCR Kit (Qiagen) and HA- and NA-specific primers targeted with M13 sequences. Each amplicon was sequenced with M13 primers in both
Enza viruses were titrated in MDCK-SIAT1 cells plated in 96-well plates at 50,000 cells per well at 18 h post infection. Neutralization assays using R3 viruses were performed to compare the fluorescence- and ELISA-based assays were done in 96-well black transparent bottom plates. Cells were plated 24 h before the experiment. Neutralization reaction was done as described above. R3 influenza neutralization assay was optimized to be performed in 384-well plate format. PB1-expressing MDCK-SIAT1 cells were washed twice with PBS, re-suspended in OptiMEM, and plated 2 h after the assay in 384-well plates at 150,000 cells ml−1 (20 μl per well). Twenty-five microliters of each neutralization mixture consisted of 2 μg ml−1 TPK-treated trypsin and equal parts of virus and 4-fold serial dilutions of antibodies. Absorbance was read at 450 nm (A450) and 650 nm (A650) with the SpectraMax. Neutralization titers were calculated using Prism as described above.

Detailed protocols are provided as Supplementary Notes 1 and 2.

Phylogenetic and evolution-based conservation analyses of influenza HA.

Nucleotide sequences of mature H1 HA (N = 25) and H3 HA (N = 26) proteins were aligned using Muscle algorithm found in Bioedit v7.2.5. Phylogenetic trees were generated using neighbor-joined methods and Kimura 2-parameter substitution model as implemented in MEGA v10. Evolution-based conservation analyses of amino acid residues in the extracellular region of H1 and H3 HA proteins was done using Consurf (http://consurf.tau.ac.il) and visualized on the atomic structures of HA proteins of A/California/07/2009 (PDB ID: 3LZG) and A/Victoria/361/2011 (PDB ID: 4WE8). To evaluate the conservation of each amino acid of H3 HA proteins isolated from influenza viruses circulating in humans, full-length H3 HA sequence from human H5N2 viruses were obtained from the GISAID database (http://platform.gisaid.org). Alignment of nucleotide sequences was performed using MAFFT v7 server-based algorithm using default settings (https://mafft.cbrc.jp/alignment/server/). After removal of redundant sequences and sequences with gaps or degenerate nucleotide bases, we obtained a dataset of 16,893 sequences. To evaluate the conservation of each amino acid residue of H3 HA proteins used in the present study have been deposited to NCBI Genbank under Accession numbers MW298519-MW298274. A reporting summary for this article is available as a Supplementary Information file. Source data are provided with this paper.

Received: 7 July 2020; Accepted: 22 February 2021; Published online: 19 March 2021

References
1. Hartfoot, R. & Webb, R. J. H5 influenza, a global update. J. Microbiol. 55, 196–203 (2017).
2. Hutchinson, E. C. & Yamauchi, Y. Understanding influenza. Methods Mol. Biol. 1836, 1–21 (2019).
3. Vijaykrishna, D. et al. The contrasting phylodynamics of human influenza B viruses. Elife 4, e05055 (2015).
4. Zost, S. J., Wu, N. C., Hensley, S. E. & Wilson, I. A. Immunodominance and antigenic variation of influenza virus hemagglutinin: implications for design of universal vaccine immunogens. J. Infect. Dis. 219, S38–S45, (2019).
5. Cakić, N., Ellis, D. & Koci, N. P. A vaccine design and delivery technologies. J. Infect. Dis. 219, S88–S96 (2019).
6. Cranik, M. C., Mascola, J. R. & Graham, B. S. Preparing for the next influenza pandemic: the development of a universal influenza vaccine. J. Infect. Dis. 219, S107–S109 (2019).
7. Crowe, J. E. Antibody determinants of influenza immunity. J. Infect. Dis. 219, S21–S29 (2019).
8. Krammer, F. & Palese, P. Universal influenza virus vaccines that target the conserved hemagglutinin stalk and conserved sites in the head domain. J. Infect. Dis. 219, S62–S67 (2019).
9. Laurie, K. L. et al. International laboratory comparison of influenza microneutralization assays for (H1N1)pdm09, (H3N2), and (H5N1) influenza viruses. J. Clin. Microbiol. 22, 957–964 (2013).
10. Donis, R. O. Antigenic analyses of highly pathogenic avian influenza a viruses. Curr. Top. Microbiol. Immunol. 385, 403–440 (2014).
11. Wei, X. et al. Antibody neutralization and escape by HIV-1. Nature 422, 307–312 (2003).
12. Pierson, T. C. et al. A rapid and quantitative assay for measuring antibody-mediated neutralization of West Nile virus infection. Virology 346, 53–65 (2006).
13. Chen, M. et al. A flow cytometry-based assay to assess RSV-specific neutralizing antibody is reproducible, efficient and accurate. J. Immunol. Methods 362, 180–184 (2010).
14. Yang, Z. Y. et al. Immunization by avian H5 influenza hemagglutinin mutants with altered receptor binding specificity. Science 317, 825–828 (2007).
15. Carnell, G. W., Ferrara, F., Grehan, K., Thompson, C. P. & Temperton, N. J. Pseudo-type-neutralized assays for influenza: a systematic analysis. Front. Immunol. 6, 161 (2015).
16. Oh, H. L. et al. An antibody against a novel and conserved epitope in the hemagglutinin 1 subunit neutralizes numerous H5N1 influenza viruses. J. Virol. 84, 8275–8286 (2010).
17. Breen, M., Nogales, A., Baker, S. F. & Martinez-Sobrido, L. Replication-competent A viruses expressing reporter genes. Viruses 8, 179 (2016).
18. Boshoms, J. D., Gong, L. I. & Baltimore, D. Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. Science 328, 1272–1275 (2010).
19. Martinez-Sobrido, L. et al. Hemagglutinin-pseudotyped green fluorescent protein-expressing influenza viruses for the detection of influenza virus neutralizing antibodies. J. Virol. 84, 2157–2163 (2010).
20. Nogales, A., Baker, S. F., Domon, W. & Martinez-Sobrido, L. Development and applications of single-cycle infectious A virus (sciAV). Virus Res. 216, 26–40 (2016).
21. Westgeest, K. B. et al. Genomewide analysis of reassortment and evolution of human influenza A(H5N2) viruses circulating between 1968 and 2011. J. Virol. 88, 2841–2857 (2014).
22. Muramoto, Y. et al. Hierarchy among viral RNA (vRNA) segments in their role in vRNA incorporation into influenza A viruses. J. Virol. 80, 2318–2325 (2006).
23. Shcherbo, D. et al. Far-red fluorescent tags for protein imaging in living tissues. Biochem. J. 418, 567–574 (2009).
24. Matrosovich, M., Matrosovich, T., Carr, J., Roberts, N. A. & Klent, H. D. Overexpression of the alpha-2,6-sialyltransferase in MDCK cells increases influenza virus sensitivity to neuraminidase inhibitors. J. Virol. 77, 8418–8425 (2003).
25. Francis, T. Jr. Vaccination against influenza. Bull. World Health Organ. 8, 725–741 (1953).
26. Smith, D. J. et al. Mapping the antigenic and genetic evolution of influenza virus. Science 305, 371–376 (2004).
27. Bedford, T. et al. Integrating influenza antigenic dynamics with molecular evolution. Elife 3, e01914 (2014).
28. Fonville, J. M. et al. Antibody landscapes after influenza virus infection or vaccination. Science 346, 996–1000 (2014).
29. Anderson, C. S., McCall, P. R., Stern, H. A., Yang, H. & Topham, D. J. Antigenic cartography of H1N1 in the World War II epidemic. Proc. Natl Acad. Sci. USA 106, 10748–10752 (2009).
30. Hobson, D., Curry, R. L., Beare, A. S. & Ward-Gardner, A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A virus. J. Hyg. 70, 767–777 (1972).
31. Meiklojoh, G., Kempe, C. H., Thalman, W. G. & Lennette, E. H. Evaluation of monovalent influenza vaccines. II. Observations during an influenza A-prime epidemic. Am. J. Hyg. 55, 12–18 (1952).
32. de Jong, J. C. et al. Haemagglutination-inhibiting antibody to influenza virus. Dev. Biol. 115, 63–73 (2003).
33. Coudeville, L. et al. Relationship between haemagglutination-inhibiting antibody titres and clinical protection against influenza: development and application of a Bayesian random-effects model. BMC Med. Res. Methodol. 10, 18 (2010).
34. Jahel, R. I. & Kilbourn, E. D. Reduction in plaque size and reduction in plaque number as differing indices of influenza-virus-antibody reactions. J. Bacteriol. 92, 1521–1526 (1966).
35. Rowe, T. et al. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. J. Clin. Microbiol. 37, 937–943 (1999).
36. Flandro, A., Garcia-Saavedra, A., Basler, C. F. & Palese, P. Chimeric influenza A viruses with a functional influenza B virus neuraminidase or hemagglutinin. J. Virol. 77, 9116–9123 (2003).
37. Horimoto, T., Iwatsuki-Horimoto, K., Hatta, M. & Kawaoka, Y. Influenza A viruses possessing type B hemagglutinin and neuraminidase: potential as vaccine components. Microbes Infect. 6, 579–583 (2004).
38. Ping, J., Lopes, T. J., Neumann, G. & Kawaoka, Y. Development of high-yield influenza B virus vaccine viruses. Proc. Natl Acad. Sci. USA 113, E8296–E8305 (2016).
67. Boyoglu-Barnum, S. et al. Quadrivalent influenza nanoparticle vaccines induce broad protection. Nature https://doi.org/10.1038/s41586-021-03365-x (2021).

68. Lin, Y., Gu, Y. & McCauley, J. W. Optimization of a quantitative micro-neutralization assay. J. Viu. Exp. https://doi.org/10.3791/54897 (2016).

69. van Baalen, C. A. et al. ViroSpot microneutralization assay for antigenic characterization of human influenza viruses. Vaccine 35, 46–52 (2017).

70. Jorquera, P. A. et al. Insights into the antigenic advancement of influenza A (H3N2) viruses, 2011-2018. Sci. Rep. 9, 2675 (2019).

71. Naito, T. et al. Generation of a genetically stable high-fidelity influenza vaccine strain. J. Virol. https://doi.org/10.1128/JVI.01073-16 (2017).

72. Georgiev, I. S. et al. Delineating antibody recognition in polyclonal sera from patterns of HIV-1 isolate neutralization. Science 340, 751–756 (2013).

73. Yassine, H. M. et al. Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection. Nat. Med. 21, 1065–1070 (2015).

74. Corbett, K. S. et al. Design of nanoparticulate group 2 influenza virus hemagglutinin stem antigens that activate unmutated ancestor B cell receptors of broadly neutralizing antibody lineages. MBio https://doi.org/10.1128/mBio.02810-18 (2019).

75. Impagliazzo, A. et al. A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen. Science 349, 1301–1306 (2015).

76. Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G. & Webster, R. G. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl Acad. Sci. USA 97, 6108–6113 (2000).

77. Ping, J. et al. Development of high-yield influenza A virus vaccine viruses. Nat. Commun. 6, 8148 (2015).

Acknowledgements
We thank Richard Webby (St. Jude Research Hospital) for influenza reverse genetics plasmids, Jesse Bloom (Fred Hutchinson Cancer Research Center) for HEK-293 cells expressing PB1 of A/WSN/1933, and Jeffrey Boyington (VRC) for help with sequence and structural analyses. This work was supported by the Intramural Research Program of the Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Electron microscopic data collection and analyses were funded by federal funds from the Frederick National Laboratory for Cancer Research, National Institutes of Health, under contract number HHSN261200800001E, and by Leidos Biomedical Research, Inc. (T.S and Y.T.).

Author contributions
Conceptualization: A.C., M.K., B.S.G.; methodology: A.C.; formal analysis: A.C., M.K.; investigation: A.C., R.A.G., B.E.F., S.F.A., J.L., C.Y., L.H., T.S., Y.T.; resources: J.E.L.; writing—original draft: A.C., M.K.; writing—review and editing: A.C., S.F.A., J.R.M., B.S.G., M.K.; supervision: A.R.M., J.R.M., B.S.G., M.K.; project administration: M.C.C.; funding acquisition: J.R.M., B.S.G.

Funding
Open Access funding provided by the National Institutes of Health (NIH).

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-21954-2.

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Peer review information Nature Communications thanks Nicholas Wu and the other anonymous reviewer(s) for their contribution to the peer review of this work.

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