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Competitive detection of influenza neutralizing antibodies using a novel bivalent fluorescence-based microneutralization assay (BiFMA)

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Abstract

Avian-derived influenza A zoonoses are closely monitored and may be an indication of virus strains with pandemic potential. Both successful vaccination and convalescence of influenza A virus in humans typically results in the induction of antibodies that can neutralize viral infection. To improve long-standing and new-generation methodologies for detection of neutralizing antibodies, we have employed a novel reporter-based approach that allows for multiple antigenic testing within a single sample. Central to this approach is a single-cycle infectious influenza A virus (sciIAV), where a functional hemagglutinin (HA) gene was changed to encode either the green or the monomeric red fluorescent protein (GFP and mRFP, respectively) and HA is complemented *in trans* by stable HA-expressing cell lines. By using fluorescent proteins with non-overlapping emission spectra, this novel bivalent fluorescence-based microneutralization assay (BiFMA) can be used to detect neutralizing antibodies against two distinct influenza isolates in a single reaction, doubling the speed of experimentation while halving the amount of sera required. Moreover, this approach can be used for the rapid identification of influenza broadly neutralizing antibodies. Importantly, this novel BiFMA can be used for any given influenza HA-pseudotyped virus under BSL-2 facilities, including highly pathogenic influenza HA isolates.

Keywords

Hemagglutinin (HA); single-cycle infectious influenza A virus (sciIAV); bivalent fluorescence-based microneutralization assay (BiFMA); neutralizing antibody (NAb); hemagglutination inhibition (HAI); virus neutralization (VN); green fluorescent protein (GFP); monomeric red fluorescent protein (mRFP)

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INTRODUCTION

Influenza A viruses reside in the wild aquatic waterfowl reservoir, but humans and other mammals are continuously affected by cross-species infection [1]. Presently two influenza A subtypes are circulating in humans (H1N1 and H3N2), which account for approximately half of the influenza clinical cases and, together with influenza B viruses, cause three to five million cases of severe illness yearly with 250,000 to 500,000 deaths worldwide [2].

Influenza A viruses are enveloped and contain eight single-stranded RNA segments of negative polarity with two major surface glycoproteins: hemagglutinin (HA), which mediates receptor binding and fusion; and neuraminidase (NA), which mediates nascent virion release [3]. Influenza A viruses are classified by their 18 HA (H1-18) and 11 NA (N1-11) antigenic variants or subtypes [4-6]. However, antigenically distinct isolates can also exist within the same subtype (referred to as drifted variants), as observed in seasonal H1N1 prior to 2009, where the pandemic H1N1 swine-origin virus displayed unique antigenicity [5, 7, 8]. A majority of influenza A virus isolated from people can readily transmit between humans via aerosolized droplets, and because airborne virus spreads so rapidly, the best mechanism to prevent disease spread is through vaccination, recommended for all non-contraindicated persons >6 months of age in a number of countries [9, 10].

Sterilizing immunity against influenza viruses can be achieved through the induction of neutralizing antibodies (NAbs), which can bind HA to prevent virus-receptor binding or virion-endosomal fusion [3]. Indeed, a four-fold vaccine-induced increase in NAbs, or a resulting >1:40 titer of protective antibodies, is clinically relevant [11, 12]. The two standard methods for evaluating humoral influenza virus inhibition are the hemagglutination inhibition (HAI) assay, which has been shown to correlate with protective immunity [13], and the virus neutralization (VN) assay. For both tests, influenza viruses are pre-incubated with serial dilutions of sera (or antibodies) before being added to erythrocytes for the HAI assay and observing red cell agglutination in a few hours [14], or to Madin-Darby canine kidney (MDCK) cell monolayers for the VN assay and observing cytopathic effect two-to-four days post-infection [15]. Both tests require intact influenza virus, which can be problematic for testing highly pathogenic influenza isolates because such viruses require high biosafety containment (e.g. BSL-3+ laboratories), although the HAI assay does not require infectious virus (e.g. can be performed using inactivated virus [16]). Furthermore, the HAI assay requires a significantly higher amount of virus per reaction (the equivalent to approximately $10^5 – 10^6$ of egg infectious $dose_{50}$, EID_{50}) [17], whereas the VN requires less virus per reaction (100 – 200 EID_{50}) [18], suggesting HAI may be less sensitive because there is more antigen for the antibodies to neutralize. Also, HAI assay readouts vary based on the amount of erythrocytes used and the subjectivity of the laboratory personnel in terms of considering the presence or absence of red cell agglutination, as well as the time when the assay is read [19]. On the other hand, the HAI is much more rapid than the VN, taking 1-2 hours rather than the 2-4 days to achieve results [15]. To obtain a VN titer more rapidly, ELISA or Western blot can be performed on infected cells the day following infection, although this adds another step that requires the use of specific antibodies against the viral antigen and qualified personnel, and that is not optimal for a large number of samples [20]. Despite their differences, both HAI and VN can only be performed against one antigenic

virus variant at a time, which is disadvantageous amid the rapid drifting of some avian H5 viruses [21]. Having a single virus per reaction also limits the detection of broadly cross-reactive influenza NAbs. Therefore, an assay for the detection of influenza NAbs that avoids the use of infectious-competent virus, is rapid, and can evaluate multiple antigenic variants of virus will help to identify and characterize laboratory-generated therapeutic NAbs and to evaluate humoral responses from influenza vaccination and infection.

An advantageous approach to detect NAbs against influenza virus with diverse HA subtypes is the HA-pseudotyped single cycle infectious influenza A virus (sciIAV) [22]. Other sciIAV engineered to delete the PB2, PB1, or NA genes have also been used to identify influenza NAbs [23-25], but changing the antigenicity of the test virus requires *de novo* virus rescue. As opposed to non-influenza virus pseudotypes, sciIAV maintains appropriate HA:NA stoichiometry, virion morphology, and once sciIAV is rescued, the backbone virus can be pseudotyped on diverse HA-expressing cells more rapidly than rescuing new viruses [22, 26, 27]. Here, we show that our previously developed fluorescence-based microneutralization assay for the detection of influenza NAbs [22] can be extended to a multiplex format to evaluate several antigenic variants of influenza virus in a single-well system. To achieve this, identical sciIAV were rescued that differ only in their fluorescent reporter gene (GFP or mRFP). We applied this bivalent fluorescence approach to demonstrate neutralization against different (heterosubtypic) and similar (homosubtypic) HA isolates. Moreover, we present evidence that BiFMA can be used to easily identify influenza broadly cross-reactive NAbs, all under less restricted BSL-2 laboratory settings. These results demonstrate the feasibility of using similar approaches to screen, in a single test, all isolates comprising vaccine formulations or multiple circulating viruses.

MATERIALS AND METHODS

Cell culture

MDCK cells (ATCC CCL-34) were maintained in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Inc.) supplemented with 10% fetal bovine serum (FBS, Atlanta biologicals), and 1% PSG (penicillin, 100 units/ml; streptomycin, 100 μg/ml; L-glutamine, 2 mM; Mediatech, Inc.). Cells were grown at 37°C in a 5% CO_2atmosphere. MDCK cells constitutively expressing influenza HA (MDCK-HA) from A/Brevig Mission/1/18 (H1N1; "1918"), A/WSN/33 (H1N1; "WSN"), A/Vietnam/1203/04 (H5N1; "Viet"), or from A/HongKong/1/1968 (H3N2; "X31") were previously described [22, 28]. MDCK-HA cells stably expressing HA from influenza A/Indonesia/5/2005 (H5N1; "Indo") were generated by co-transfecting the pCAGGS HA-expressing Indo plasmid and pCB7 (3:1 ratio) for eukaryotic expression of HA and Hygromycin B resistance, respectively [22, 29, 30]. MDCK-HA cells were cultured in DMEM/10% FBS/1% PSG supplemented with 200 μg/ml Hygromycin B (Corning). After viral infections, cells were maintained at 37°C in 5% CO_2atmosphere in DMEM containing 0.3% bovine serum albumin (BSA), 1% PSG, and 1 μg/ml tosyl-sulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) [31].

Viruses and plasmids

Influenza WSN reverse genetics plasmids [32] and plasmids pPolI HA(45)GFP(80) and pPolI HA(45)mRFP(80) used to generate WSN sciIAV [27] have been previously described. HA-pseudotyped sciIAV (GFP or mRFP) were propagated in MDCK-HA cells (MOI 0.001, 37°C, 3 days) and titrated on MDCK-HA cells (fluorescent focus units, FFU) [30]. Terminology hereafter referring to WSN HA pseudotyped GFP-expressing sciIAV is referred to pWSN sciIAV GFP, for example.

Antibodies

Mouse monoclonal antibodies against WSN HA (2G9) [33], 1918 HA (39E4) and Viet HA (23E6) [22] have been previously described. The pan anti-H1 (6F12) [34], and pan anti-Group 1 (KB2 [35] and GG3 [36]) mouse monoclonal antibodies were kindly provided by Dr. Peter Palese (Icahn School of Medicine at Mount Sinai). Mouse monoclonal antibody against Viet HA (NR-2730) and goat polyclonal anti-X31 NR-3118 were obtained from BEI Resources (NIAID, NIH). Mouse polyclonal anti-Indo HA sera (3xIndo) was obtained from mice immunized three times, at two-weeks intervals, with 2 μg of recombinant Indo H5 (BEI Resources, NR-10511) adjuvanted with CpG oligonucleotides (ODN-1826), as previously described [37]. NAbs are summarized in **Appendix 1**.

Growth kinetics of sciIAV

Multicycle growth analyses were performed by infecting (MOI 0.001) confluent monolayers of parental or MDCK-HA cells (5×10^5 cells, 12-well plate format, triplicates) with sciIAV [22]. At indicated times post-infection, GFP expression was assessed by fluorescence microscopy, and viral titers in tissue culture supernatants (TCS) were measured by evaluating FFU/ml in a focus assay. Briefly, confluent wells of MDCK-HA cells (5×10^4 cells, 96-well plate format, triplicates) were infected with 10-fold serial dilutions of TCS. Eighteen hours post-infection, cells were washed with 1X PBS and foci were visualized using a fluorescence microscope and enumerated. Mean value and standard deviation were calculated using Microsoft Excel software.

Immunofluorescence assay

For the characterization of MDCK-HA cells, confluent monolayers of parental or MDCK-HA cells (10^5 cells, 48-well plate format) were fixed with 4% paraformaldehyde and stained as previously described [26, 30]. Dilutions for primary antibodies are as follows: 6F12, GG3, and KB2 (5 μg/ml); 2G9 (1.5 μg/ml); 39E4, 23E6, NR-2730 (1 μg/ml); NR-3118 (1:1000 dilution). Primary antibodies were detected with FITC-conjugated secondary anti-mouse (1:140, Dako) or anti-goat (1:200, Jackson ImmunoResearch) antibodies and 4’,6’-diamidino-2-phenylindole (DAPI; Research Organics). HA-expressing MDCK cells were visualized using a fluorescence microscope, photographed (Cooke Sensicam QE) with a 20X objective (200X actual), and colored and merged using Adobe Photoshop CS4 (v11.0) software. Representative images from representative fields are shown.

Fluorescence-based microneutralization assays

Triplicate wells (96-well plate format), containing 200 FFU of HA-pseudotyped WSN sciIAV were pre-incubated with serial (1:2) dilutions of monoclonal or polyclonal antibodies (starting concentrations indicated in each experiment) for one hour at room temperature. Virus/antibody mixtures were then used to infect confluent monolayers of MDCK X31-HA cells (4×10^4 cells/well, 96-well plate format). MDCK X31-HA cells were used because the NAbs tested did not have reactivity against group 2 X31 HA. Twenty-four hours post-infection, cells were washed with 1X PBS and analyzed for viral infection using a fluorescence plate reader (DTX-880, Beckman Coulter) as previously described [22, 38, 39]. Percent neutralization was determined as previously described [29]. Values from individual replicates that were greater than 100% or less than 0% were set to 100% or 0%, respectively. Sigmoidal dose-response curves from neutralization data and the NAb concentrations at which 50% virus is neutralized (NT50) were calculated by GraphPad Prism software.

Bivalent microneutralization assays in sciIAV GFP and sciIAV mRFP co-infected MDCK-HA cells were performed as above, but the amount of virus was adjusted to achieve uniform co-infection in the absence of antibody. To determine virus quantities, MDCK-HA cells were co-infected with five, two-fold serial dilutions (starting inoculum of 2,000 FFU/well) of mRFP and GFP viruses in triplicate, testing all combinations (data not shown), and the conditions that provided the highest signal above background fluorescence at 24 hours post-infection for both viruses was used. BiFMA assays were performed as described above and previously [22, 29, 38, 39].

RESULTS AND DISCUSSION

Characterization of GFP or mRFP-expressing sciIAV

HA-deficient sciIAV contain an HA gene segment where the wild-type (WT) non-coding regions and packaging signals flank the open reading frame of GFP or mRFP (HA[45]GFP[80] and HA[45]mRFP[80], respectively; **Fig. 1A**), and HA *trans*-complementation is achieved through growth in MDCK-HA cells [27]. Reverse genetics plasmids were used to rescue recombinant WSN sciIAV that contain the modified HA genes (sciIAV GFP and sciIAV mRFP) as previously described [27, 32]. To compare effects of differential fluorescent protein expression on virus growth, parallel multicycle growth kinetics were performed in parental or WSN-HA MDCK cells (**Fig. 1B & 1C**). Virus collected from TCS at various times post-infection indicates that sciIAV GFP and sciIAV mRFP grow with similar kinetics (**Fig. 1B**). Although both cell types were initially permissive to the low MOI (0.001) infection, only MDCK-HA cells supported the spread of virus, visualized by fluorescent focus formation (**Fig. 1C**).

Replication-deficient influenza viruses that express reporter proteins are useful in studying virus reassortment [26, 40, 41] and entry [42], and have been adapted to high throughput methodologies to screen for host factors involved in replication [43] and to detect NAbs [22, 23, 44]. To determine the amenability of sciIAV mRFP to a microneutralization assay incorporating a fluorescence plate reader, sciIAV GFP [22] and sciIAV mRFP were used in individual fluorescent microneutralization assays with the anti-WSN NAb 2G9. Importantly,

the fluorescence expression of infected cells shows a dose-dependent increase of GFP or mRFP as the NAb is diluted (**Fig. 1D & 1E**). Importantly, both sciIAV GFP and mRFP variants were neutralized to similar levels with 2G9. To test the feasibility of co-infecting with sciIAV GFP and sciIAV mRFP, an additional microneutralization assay was performed. When equivalent amounts of sciIAV GFP and sciIAV mRFP were incubated with serial dilutions of 2G9 and used to co-infect MDCK-HA cells, the sciIAV GFP and mRFP were neutralized to similar levels (**Fig. 1F**). The neutralization capacity of 2G9 was reduced by half in this bivalent assay, likely because the amount of virus used in total was doubled (**Appendix 2A**). These results suggest that different fluorescent reporter genes do not affect the replication capability of sciIAV or the ability to neutralize the same HA-pseudotyped sciIAV in single or in co-infection experiments.

Pseudotyping sciIAV with antigenically distinct HA

MDCK-HA cells infected with sciIAV will produce virus particles carrying the antigenicity of the host cell [22, 26, 30]. To demonstrate antigenic differences of the HA isolates tested for further studies herein, MDCK-HA cells were stained with various antibodies and evaluated by immunofluorescence assay (**Fig. 2A**). Monoclonal antibodies generated against the HA of WSN (2G9), 1918 (38E4), or Viet (NR-2730) specifically reacted with MDCK cells expressing WSN-HA, 1918-HA, or Viet-HA, respectively. Since we could not acquire an antibody that specifically reacted with the HA from Indo, the 29E3 monoclonal antibody generated against the HA of Viet was used, which reacted with both MDCK Indo-HA and Viet-HA cells. Due to the vast antigenic difference of the group 2 HA of X31, a polyclonal anti-H3 antibody only reacted with MDCK X31-HA cells. Lastly, and as expected, the stalk-reactive anti-H1 antibody 6F12 reacted with only MDCK WSN-HA and 1918-HA, and the stalk-reactive anti-group 1 HA antibodies GG3 and KB2 reacted with WSN-HA, 1918-HA, Viet-HA and Indo-HA (group 1), but not group 2 (X31-HA) MDCK cells (**Fig. 2A and Appendix 1**).

Previous work performed by us [27, 30] and others [45] has shown that sciIAV replicates similarly, albeit 0.5 – 1 $\log_{10}$lower than WT virus in MDCK-HA cells. To evaluate if diverse HA isolates with distinct antigenicity affected replication of sciIAV, multicycle growth kinetics were performed (**Fig 2B & 2C**). GFP expression from sciIAV infected cells shows that only cells expressing HA could complement the virus and allow spreading and focus formation (**Fig. 2B**). Virus quantified from TCS at various times post-infection demonstrates that matched WSN-HA supported sciIAV growth most rapidly, whereas all remaining MDCK-HA cells, regardless of the divergence of HA, complemented virus to similar levels (**Fig. 2C**).

Using HA pseudotypes from both group 1 and group 2 will better limit non-specific neutralization. Phylogenetic analysis of the HA isolates used here (**Fig. 2D**) clearly demonstrates this relationship, indicating the vast difference of the BiFMA substrate MDCK X31-HA cells from all other HA isolates at the amino acid level, and furthermore, the relatedness of the two H5 isolates is much higher than the two H1 isolates.

BiFMA can be used to specifically identify neutralization of viruses with different HA subtypes

To test if BiFMA could be used to detect H5 or H1 NAbs in co-infections, the assay was first used to evaluate neutralization during mono-infections (**Fig. 3**). When pViet sciIAV GFP (H5N1) was used in a fluorescence-based microneutralization assay with NR-2730 (**Fig. 3A**) or 2G9 (**Fig. 3B**), the virus was only neutralized by the H5-specific NAb. As expected, the opposite results occurred when pWSN sciIAV mRFP (H1N1) was used with NR-2730 (**Fig. 3D**) or 2G9 (**Fig. 3E**). To query whether specificity was lost when NAbs were used in polyclonal preparations (as is the case for sera), both NR-2730 and 2G9 were used together in mono-virus microneutralization assays (**Fig. 3C & 3F**), which gave similar results as the Nabs individually. Importantly, pViet and pWSN sciIAV co-infections produce similar neutralization results as the mono-infections (**Fig. 3G-3I**). Although comparing the neutralization titers (**Appendix 2B**) shows a difference in some cases, BiFMA is able to clearly identify NAbs against viruses pseudotyped with distinct HA subtypes similar to observations with individual NAb and virus preparations.

BiFMA can specifically determine neutralization of viruses within the same HA subtype

As evidenced by the differences of pandemic 2009 H1N1 and previously circulating seasonal H1N1 viruses [5, 7, 8], vast antigenic changes can be observed within the same influenza subtype. To evaluate if BiFMA can be used to detect NAbs specific to a given isolate within the same subtype, p1918 sciIAV GFP (H1N1) and pWSN sciIAV mRFP (H1N1) were evaluated with monoclonal antibodies (**Fig. 4**). When comparing mono-infections, as above, 39E4 was able to neutralize p1918 and not pWSN sciIAV (**Figs. 4A and 4D**, respectively). Likewise, 2G9 specifically neutralized pWSN but not p1918 sciIAV (**Figs. 4E and 4B**, respectively). Additionally, combining the two antibodies together resulted in similar neutralization as observed for individual Nabs (**Figs. 4C and 4F**). Despite the similarity of the two H1N1 HA isolates tested (**Fig. 2**), the results obtained with BiFMA were similar to the mono-infections (**Fig. 4G-4I; Appendix 2C**).

Highly pathogenic avian influenza H5N1 viruses of are rapidly evolving, which has resulted in the generation of multiple distinct clades of H5 HA [21]. Although Viet (clade 1) and Indo (clade 2.1.3.2) are more related than the two H1N1 viruses 1918 and WSN, some antigenic differences remain (**Fig. 2**). Therefore, the amenability of BiFMA to detect NAbs specific for actively diverging clades of H5N1 HA was tested (**Fig. 5**). In agreement with the immunofluorescence results (**Fig. 1D**), NR-2730 only neutralized pViet sciIAV GFP and not pIndo sciIAV mRFP (**Figs. 5A and 5D**, respectively). Since the antibody 23E6 used to detect the HA of MDCK Indo-HA cells was not able to neutralize pIndo sciIAV (data not shown), we generated hyperimmune mouse sera by vaccinating mice with recombinant Indo HA (3xIndo). Although 3xIndo sera could neutralize pViet, the sera was much more potent against pIndo in mono-infections (**Figs. 5B and 5E**), and as expected, combining these sera and antibodies did not change the resulting neutralization (**Figs. 5C and 5F**). Lastly, combining both H5 pseudotyped viruses for BiFMA showed comparable results as observed with mono-infections (**Figs. 5G-5I; Appendix 2D**), supporting that this bivalent approach can accurately detect specific neutralization of H5 influenza viruses during co-infection.

Detection of stalk-reactive NAbs using BiFMA

Most NAb epitopes described for influenza are located proximal to the receptor binding domain found in the globular head of HA [3, 46]. In contrast, the stalk region of HA has a higher degree of conservation, and antibodies have been identified that bind to homosubtypic drifted variants, multiple subtypes within the same group, across groups, and even between both influenza type A and B viruses (see [46] for review). Stalk-reactive NAbs cannot be identified using HAI because this assay specifically identifies Abs that occlude HA from binding to sialylated receptors on erythrocytes [47, 48]. Similarly, VN assays are less effective in identifying stalk-reactive antibodies, requiring 100 – 1,000 times more NAb than pseudovirus assays [49, 50]. Previously, we were able to identify stalk-reactive NAbs using a fluorescence-based approach [38]; thus, we tested the ability of BiFMA to detect stalk-reactive NAbs (**Fig. 6**). Using the pan anti-H1 antibody 6F12, in both homosubtypic (e.g., H1 vs H1, H5 vs H5) and heterosubtypic (e.g., H1 vs H5) BiFMA, 6F12 was only able to neutralize pWSN and p1918 sciIAV (**Figs. 6A-6D**). In contrast, when the pan anti-group 1 antibodies KB2 or GG3 were used, both H1 and H5 pseudotyped viruses were neutralized, albeit to different levels (**Figs. 6E-6L; Appendix 2E**). The differences in neutralization potency may be due to varying degrees of epitope conservation between isolates, but most importantly, the identity of each virus was correctly confirmed by neutralization, in agreement with previous results [34-36].

CONCLUSIONS

Although a major contribution of immunity against influenza virus infections is driven by cell-mediated responses, prior induction of or therapeutic treatment with NAbs can limit virus infection [46, 51]. Sterilizing immunity can be achieved through NAb generation, whereas immunity against heterosubtyic influenza viral infection can rely on cellular responses, which is often concomitant with morbidity and transient virus replication [52, 53]. Improving the current methodologies to detect NAbs is needed, especially in areas with live poultry markets and farms that are affected by highly pathogenic avian influenza viruses of the H5, H7, H9 and H10 subtypes [54-57]. However, improvement of sero surveillance is hampered because BSL-3 facilities are needed to handle highly pathogenic influenza strains. Moreover, conventional assays to detect neutralizing antibody responses can only be performed against individual viruses and are not useful for the detection of broadly neutralizing antibodies.

To overcome these deficiencies, we demonstrate that the BiFMA can rapidly, quantitatively, and safely detect NAbs in a multiplex format without the need for secondary reagents or assays for virus detection. Although the present work details the use of sciIAV pseudotyped through propagation in stable expressing cell lines, MDCK cells transiently transfected with HA protein can also be used to pseudotype the virus to more rapidly respond to emerging viruses [30]. Transfection efficiencies may however be a limiting factor for pseudotype virus production. This approach substantially improves on previous mono-infection VN assays by reducing the time and sample volume needed by half when screening against multiple HA isolates. Moreover, this methodology could be used to easily identify stalk-reactive NAbs. By using this HA-pseudotyped sciIAV approach, the BiFMA can precisely detect NAbs that

react with influenza particles, whereas another multiplexing approach relies on pseudotyped lentiviruses [58]. Other reporter (fluorescent or luminescent) proteins may improve assay sensitivity or the number of viruses that can be tested simultaneously, and future development could be implemented to identify antivirals, evaluate virus competition and fitness, or to safely characterize divergent tropism in infected laboratory animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- We have developed a bivalent fluorescence assay to identify influenza neutralizing antibodies
- The bivalent nature of the approach reduces by half the time, reagents, and sera needed
- Direct competition of antibody neutralization against disparate HAs can be evaluated
- Rapid and safe methodology amenable to facilities lacking BSL-3 capabilities
- Can be used to identify and characterize influenza broadly neutralizing antibodies

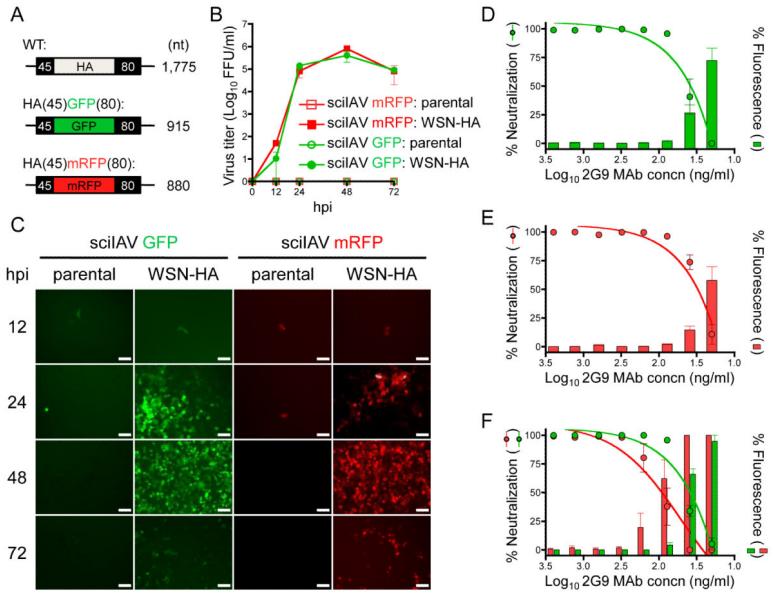

Figure 1. Characterization of GFP or mRFP expressing sciIAV

A) Schematic representation of HA segments from WT, sciIAV GFP, or sciIAV mRFP: Conservation of the non-coding regions (32 and 45 nt for 3' and 5' ends, respectively; black lines) plus the packaging signals (black boxes, nt length indicated) from WT HA allow for incorporation of the GFP or mRFP vRNA-like segment into sciIAV. Length of vRNA segments are indicated on the right. Not drawn to scale. B-C) Multicycle growth kinetics of sciIAV GFP and mRFP: Triplicate confluent monolayers of parental or WSN-HA MDCK cells were infected with sciIAV (MOI 0.001). At various times post-infection, TCS were collected for titration in MDCK-HA cells (**B**), and fluorescence images were captured (**C**) (X10 objective, X100 actual representative fields). Scale bars, 40μM; hpi, hours post-infection. Data represent means +/−SD of the results determined for triplicates. **D-F) Fluorescence-based microneutralization assay:** Two hundred FFU of sciIAV containing GFP (**D**) or mRFP (**E**) were incubated with two-fold serial dilutions of monoclonal antibody 2G9 prior to infection of MDCK X31-HA cells. Twenty-four hours post-infection, fluorescence was measured using a plate reader with filters for GFP or mRFP, and used to determine percent fluorescence (columns) and percent neutralization (circles and sigmoidal dose-response curves). Simultaneous co-infection with equivalent fluorescent units of sciIAV GFP and sciIAV mRFP (**F**) were similarly performed. Percent fluorescence values were normalized to virus in the absence of antibody (100%). Data represent the means +/−SEM of the results determined for triplicates.

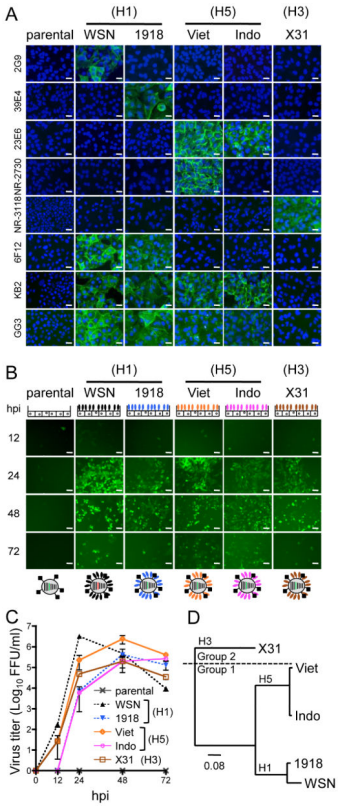

Figure 2. Characterization of MDCK-HA cell lines used to generate pseudotyped sciIAV

A) Cell surface expression of antigenically distinct HAs: MDCK cells lacking (parental) or expressing HA from H1, H5, or H3 subtypes were fixed and stained with indicated antibodies and counterstained with DAPI. 2G9, anti-WSN MAb; 39E4, anti-1918 MAb; 23E6 and NR-2730, anti-Viet MAbs; NR-3118, anti-H3 PAb; 6F12, pan anti-H1 stalk MAb; KB2 and GG3, pan anti-group 1 HA MAbs. Representative images with X20 objective (X200 actual) are shown. Scale bars, 20 μM. B) Multicycle growth analysis of sciIAV in parental and MDCK-HA cells: Parental and HA-expressing MDCK cells were infected (MOI 0,001) with GFP-containing sciIAV. At various times post-infection GFP was visualized by fluorescence microscopy (X10 objective, X100 actual representative images). Scale bars, 40μM; hpi, hours post-infection. Schematic representations of various HA isolates by color (top) WSN (black), 1918 (blue), Viet (orange), Indo (magenta), X31 (brown), and the pseudotyped sciIAV particles produced (bottom). **C) Virus titration:** TCS from cells infected in panel **B** were collected for virus titration in MDCK-HA cells. Data represent means +/−SD of the results determined in triplicates. **D) Phylogeny of HA isolates:** HA amino acid alignments were performed with Archeaopteryx, and phylogenetic trees constructed using FigTree. Group 1 and 2 HAs are separated by a dotted line, tree rooted on X31 HA.

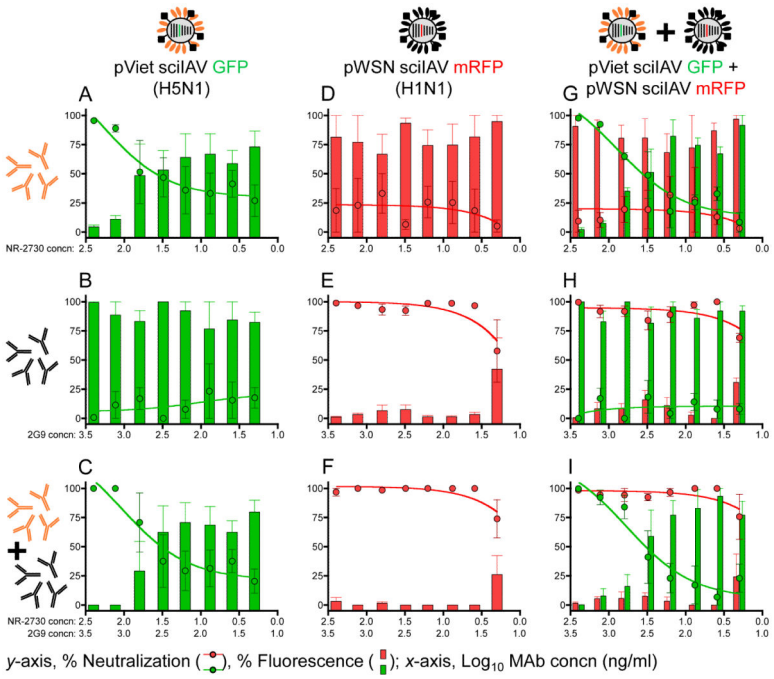

Figure 3. Heterosubtypic specific neutralization detected by BiFMA

Schematic representations of sciIAV per column (top) and NAbs per row (left) with a color code scheme to indicate NAb specificity. A-F) Fluorescence-based microneutralization of sciIAV mono-infections: Preparations of sciIAV GFP pseudotyped with Viet HA (orange; **A-C**), or sciIAV mRFP pseudotyped with WSN HA (black; **D-E**) were incubated with NAbs NR-2730 (orange; **A and D**), 2G9 (black; **B and E**) or both (**C and F**). G-I) BiFMA analysis to simultaneously evaluate NAbs against H1 and H5 influenza viruses: Co-infection with equivalent fluorescent units of pViet sciIAV GFP and pWSN sciIAV mRFP pre-treated with NR-2730 (**G**), 2G9 (**H**), or both NAbs (**I**). Percent fluorescence values were normalized to virus in the absence of Ab (100%). Data represent the means +/−SEM of the results determined for triplicates. Columns represent percent fluorescence; circles represent percent neutralization, values used to plot sigmoidal dose-response curves.

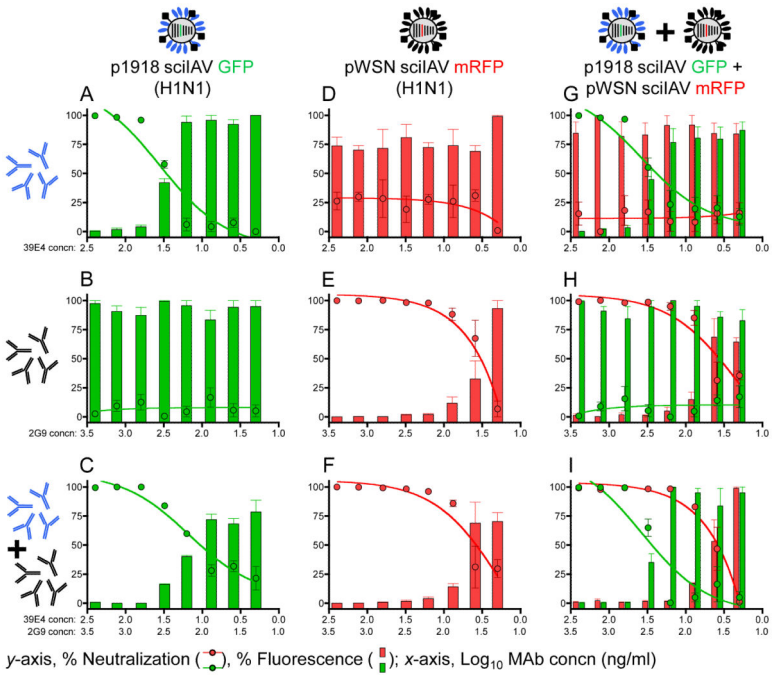

Figure 4. Homosubtypic (H1N1) specific neutralization detected by BiFMA

Schematic representations of sciIAV and NAbs organized as in **Fig. 3**. A-F) Fluorescence based microneutralization of sciIAV mono-infections: p1918 sciIAV GFP (blue; **A-C**), or pWSN sciIAV mRFP (black; **D-E**) were incubated with NAbs 39E4 (blue; **A and D**), 2G9 (black; **B and E**) or both NAbs (**C and F**). G-I) BiFMA analysis to simultaneously evaluate NAbs against two H1 influenza viruses: Co-infection with equivalent fluorescent units of p1918 and pWSN sciIAV pre-treated with 39E4 (**G**), 2G9 (**H**), or both NAbs (**I**). Percent fluorescence values were normalized to virus in the absence of Ab (100%). Data represent the means +/−SEM of the results determined for triplicates. Columns represent percent fluorescence; circles represent percent neutralization, values used to plot sigmoidal dose-response curves.

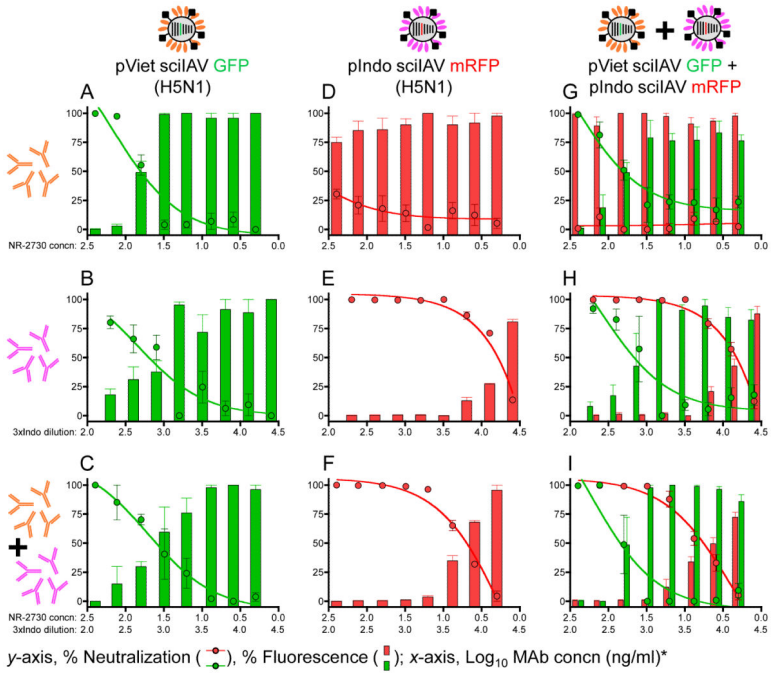

Figure 5. Homosubtypic (H5N1) specific neutralization detected by BiFMA

Schematic representations of sciIAV and NAbs organized as in **Fig. 3**. A-F) Fluorescence based microneutralization of sciIAV mono-infections: pVIet sciIAV GFP (orange; **A-C**), or pIndo sciIAV mRFP (magenta; **D-E**) were incubated with NAb NR-2730 (orange; **A and D**), sera from mice hyper-immune to Indo HA (3xIndo, magenta; **B and E**) or both NAb and sera mixed (**C and F**). G-I) BiFMA analysis to simultaneously evaluate NAbs against two H1 influenza viruses: Co-infection with equivalent fluorescent units of pVIet and pIndo sciIAV pre-treated with NR-2730 (**G**), 3xIndo (**H**), or NR-2730 and 3xIndo (**I**). Percent fluorescence values were normalized to virus in the absence of Ab (100%). Data represent the means +/−SEM of the results determined for triplicates. Columns represent percent fluorescence; circles represent percent neutralization, values used to plot sigmoidal dose-response curves. Note, 3xIndo concentration indicated reciprocal sera dilution, starting at 1:200.

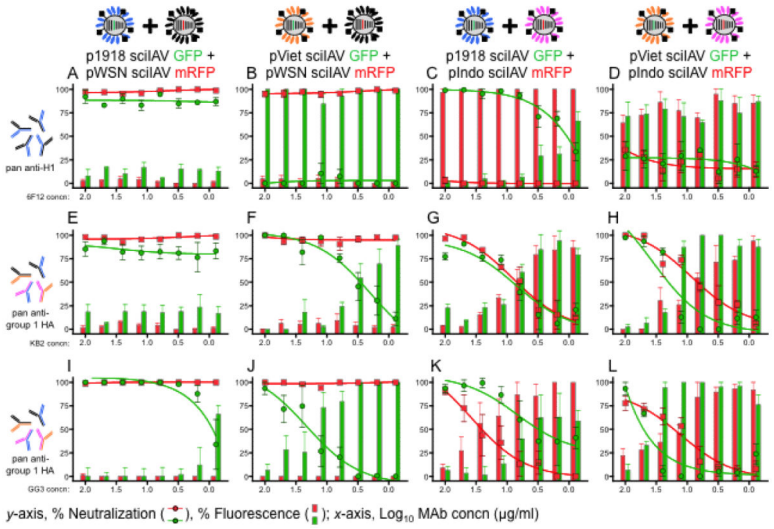

Figure 6. BiFMA detects stalk-reactive NAbs

Preparations of sciIAV pseudotyped with HA from 1918 (blue), WSN (black), Viet (orange) or Indo (magenta), were used for the indicated bivalent infections with equivalent fluorescent units of sciIAV GFP and sciIAV mRFP. Schematic representation of virus co-infections (top), and stalk-reactive antibodies (left). Note, each NAb is monoclonal, different color code schemes are used for depiction of reactivity. **A-D)** Neutralization using the stalk-reactive pan-H1 antibody 6F12 (**A-D**) and the pan-group 1 antibodies KB2 (**E-H**) or GG3 (**I-L**). Percent fluorescence values were normalized to virus in the absence of Ab (100%). Data represent the means +/−SEM of the results determined for triplicates. Columns represent percent fluorescence; circles represent percent neutralization, values used to plot sigmoidal dose-response curves.