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Using zebrafish models of human influenza A virus infections to screen antiviral drugs and characterize host immune cell responses --Manuscript Draft--

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Abstract:	Each year, seasonal influenza outbreaks profoundly affect societies worldwide. In spite of global efforts, influenza remains an intractable healthcare burden. The principle strategy to curtail infections is yearly vaccination. In individuals who have contracted influenza, antiviral drugs can mitigate symptoms. There is a clear and unmet need to develop alternative strategies to combat influenza. Several animal models have been created to model host-influenza interactions. Here, protocols for generating zebrafish models for systemic and localized human influenza A virus (IAV) infection are described. Using a systemic IAV infection model, small molecules with potential antiviral activity can be screened. As a proof-of-principle, a protocol that demonstrates the efficacy of the antiviral drug Zanamivir in IAV-infected zebrafish is described. It shows how disease phenotypes can be quantified to score the relative efficacy of potential antivirals in IAV-infected zebrafish. In recent years, there has been increased appreciation for the critical role neutrophils play in the human host response to influenza infection. The zebrafish has proven to be an indispensable model for the study of neutrophil biology, with direct impacts on human medicine. A protocol to generate a localized IAV infection in the Tg(mpx:mCherry) zebrafish line to study neutrophil biology in the context of a localized viral infection is described. Neutrophil recruitment to localized infection sites provides an additional quantifiable phenotype for assessing experimental manipulations that may have therapeutic applications. Both

	zebrafish protocols described faithfully recapitulate aspects of human IAV infection. The zebrafish model possesses numerous inherent advantages, including high fecundity, optical clarity, amenability to drug screening, and availability of transgenic lines, including those in which immune cells such as neutrophils are labeled with fluorescent proteins. The protocols detailed here exploit these advantages and have the potential to reveal critical insights into host-IAV interactions that may ultimately translate into the clinic.
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TITLE:

Using zebrafish models of human influenza A virus infections to screen antiviral drugs and characterize host immune cell responses

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SHORT ABSTRACT:

Systemic and localized zebrafish infection models for human influenza A virus are demonstrated. Using a systemic infection model, zebrafish can be used to screen antiviral drugs. Using a localized infection model, zebrafish can be used to characterize host immune cell responses.

LONG ABSTRACT:

Each year, seasonal influenza outbreaks profoundly affect societies worldwide. In spite of global efforts, influenza remains an intractable healthcare burden. The principle strategy to curtail infections is yearly vaccination. In individuals who have contracted influenza, antiviral drugs can mitigate symptoms. There is a clear and unmet need to develop alternative strategies to combat influenza. Several animal models have been created to model host-influenza interactions. Here, protocols for generating zebrafish models for systemic and localized human influenza A virus (IAV) infection are described. Using a systemic IAV infection model, small molecules with potential antiviral activity can be screened. As a proof-of-principle, a protocol that demonstrates the efficacy of the antiviral drug Zanamivir in IAV-infected zebrafish is described. It shows how disease phenotypes can be quantified to score the relative efficacy of potential antivirals in IAV-infected zebrafish. In recent years, there has been increased appreciation for the critical role neutrophils play in the human host response to influenza infection. The zebrafish has proven to be an indispensable model for the study of neutrophil biology, with direct impacts on human medicine. A protocol to generate a localized IAV infection in the *Tg(mpx:mCherry)* zebrafish line to study neutrophil biology in the context of a localized viral infection is described. Neutrophil recruitment to localized infection sites provides an additional quantifiable phenotype for assessing experimental manipulations that may have therapeutic applications. Both zebrafish protocols described faithfully recapitulate aspects of human IAV infection. The zebrafish model possesses numerous inherent advantages, including high fecundity, optical clarity, amenability to drug screening, and availability of transgenic lines, including those in which immune cells such as neutrophils are labeled with fluorescent proteins. The protocols detailed here exploit these advantages and have the potential to reveal critical insights into host-IAV interactions that may ultimately translate into the clinic.

INTRODUCTION:

According to the World Health Organization (WHO), influenza viruses infect 5-10% of adults and 20-30% of children annually and cause 3-5 million cases of severe illness and up to 500,000 deaths worldwide¹. Yearly vaccinations against influenza remain the best option to prevent

disease. Efforts like the WHO Global Action Plan have increased seasonal vaccine use, vaccine production capacity, and research and development into more potent vaccine strategies in order to reduce morbidity and mortality associated with seasonal influenza outbreaks². Antiviral drugs like neuraminidase inhibitors (e.g. Zanamivir and Oseltamivir) are available in some countries and have proven effective in mitigating symptoms, when administered within the first 48 hours of onset³⁻⁵. Despite global efforts, containment of seasonal influenza outbreaks remains a formidable challenge at this time, as influenza virus antigenic drift often exceeds current abilities to adapt to the changing genome of the virus⁶. Vaccine strategies targeting new strains of virus must be developed in advance and are sometimes rendered less than optimally effective due to unforeseen changes in the types of strains that eventually predominate in an influenza season. For these reasons, there is a clear need to develop alternative therapeutic strategies for containing infections and reducing mortalities. By achieving a better understanding of the host-virus interaction, it may be possible to develop new anti-influenza medicines and adjuvant therapies^{7,8}.

The human host-influenza A virus (IAV) interaction is complex. Several animal models of human IAV infection have been developed in order to gain insight into the host-virus interaction, including mice, guinea pigs, cotton rats, hamsters, ferrets, and macaques⁹. While providing important data that have enhanced the understanding of host-IAV dynamics, each model organism possesses significant drawbacks that must be considered when attempting to translate the findings into human medicine. For example, mice, which are the most widely used model, do not readily develop IAV-induced infection symptoms when infected with human influenza isolates⁹. This is because mice lack the natural tropism for human influenza isolates since mouse epithelial cells express α -2,3 sialic acid linkages instead of the α -2,6 sialic acid linkages expressed on human epithelial cells¹⁰. The hemagglutinin proteins present in human IAV isolates favorably bind and enter host cells bearing α -2,6 sialic acid linkages through receptor-mediated endocytosis^{9,11-13}. As a consequence, it is now accepted that in developing mouse models for human influenza, care must be taken to pair the appropriate strain of mouse with the appropriate strain of influenza in order to achieve disease phenotypes that recapitulate aspects of the human illness. In contrast, epithelial cells in the upper respiratory tract of ferrets possess α -2,6 sialic acid linkages that resemble human cells¹⁴. Infected ferrets share many of the pathological and clinical features observed in the human disease, including the pathogenicity and transmissibility of human and avian influenza viruses^{14,15}. They are also highly amenable to vaccine efficacy trials. Nevertheless, the ferret model for human influenza has several disadvantages principally related to their size and cost of husbandry that make acquisition of statistically significant data challenging. In addition, ferrets have previously displayed differences in drug pharmacokinetics, bioavailability, and toxicity that make testing efficacy difficult. For example, ferrets exhibit toxicity to the M2 ion channel inhibitor amantadine¹⁶. Thus, it is clear that in choosing an animal model to study questions about human IAV infections, it is important to consider its inherent advantages and limitations, and the aspect of the host-virus interaction that is under investigation.

The zebrafish, *Danio rerio*, is an animal model that provides unique opportunities for investigating microbial infection, host immune response, and potential drug therapies¹⁷⁻²⁸. The

presence of α -2,6-linked sialic acids on the surface of cells in the zebrafish suggested its susceptibility to IAV, which was borne out in infection studies and imaged *in vivo* using a fluorescent reporter strain of IAV¹⁹. In IAV-infected zebrafish, increased expression of the antiviral *ifnphi1* and *mxr* transcripts indicated that an innate immune response had been stimulated, and the pathology displayed by IAV-infected zebrafish, including edema and tissue destruction, was similar to that observed in human influenza infections. Furthermore, the IAV antiviral neuraminidase inhibitor Zanamivir limited mortality and reduced viral replication in zebrafish¹⁹.

In this report, a protocol for initiating systemic IAV infections in zebrafish embryos is described. Using Zanamivir at clinically relevant doses as a proof-of-principle, the utility of this zebrafish IAV infection model for screening compounds for antiviral activity is demonstrated. In addition, a protocol for generating a localized, epithelial IAV infection in the zebrafish swim bladder, an organ that is considered to be anatomically and functionally analogous to the mammalian lung^{21,29-31}, is described. Using this localized IAV infection model, neutrophil recruitment to the site of infection can be tracked, enabling investigations into the role of neutrophil biology in IAV infection and inflammation. These zebrafish models complement existing animal models of human IAV infections and are particularly useful for testing small molecules and immune cell responses because of the possibility of enhanced statistical power, capacity for moderate- to high-throughput assays, and the abilities to track immune cell behavior and function with light-microscopy.

PROTOCOL:

All work should be performed using biosafety level 2 (or BSL2) standards described by the U.S. Centers for Disease Control (CDC) and in accordance with directives established by Institutional Animal Care and Use Committees (IACUC). Please confer with the appropriate officials to ensure safety and compliance.

1. Zebrafish care and maintenance

1.1. Spawn zebrafish and collect the required number of embryos for the experiments. When necessary, mass breeding tanks, like those described by Adatto et al.³², can be employed to collect large numbers of developmentally-staged embryos.

1.2. Allow embryos to develop until desired developmental stage (48 h post-fertilization for a systemic IAV infection [section 3] or 5 d post-fertilization for a localized, swim bladder infection [section 4]) in deep Petri dishes at low density (<100 embryos/dish) at 28 °C in sterile egg water containing 60 μ g/mL sea salt (e.g., Instant Ocean) in distilled water.

1.2.1. Remove dead embryos with plastic transfer pipets and change egg water daily to ensure optimal health and development.

2. Preparation of materials and reagents

2.1. Prepare a 4 mg/mL stock solution of Tricaine-S (adjust pH to 7.0-7.4) in distilled water

and autoclave. Store at 4 °C.

2.2. Pull borosilicate glass capillaries with filaments using a micropipette puller (e.g. Micropipette Puller with the following settings: pressure setting =100, heat = 550, pull = [no value], velocity = 130, time = 110).

NOTE: Prior to trimming the needle, the length from where the needle begins to taper to the tip of the needle would be approximately 12-15 mm. This can vary, however, based on preference and application. A longer, more gradual taper may be more preferable because of the increased ability to pierce the embryo and the reduced risk of the needle bending or breaking.

2.3. Melt 2 g of agarose in 100 mL egg water using a microwave. Make plates on which to line up and inject the embryos by pouring melted agarose into deep Petri dishes and allowing it to solidify.

2.4. Make a 10 mg/mL stock of Zanamivir in nuclease-free water. Aliquot and freeze at -20 °C.

2.5. Melt 0.5 g agarose in 50 mL egg water using a microwave to make embryo mounting agarose. Maintain in water bath at 50 °C until ready to use during imaging.

3. Systemic IAV Infection (48 h post-fertilization)

CAUTION: All research personnel should consult with their supervisors and physicians regarding vaccination prior to starting work with IAV.

3.1. Manually dechorionate embryos on the day of the experiment using #5 forceps. Take care to remove and euthanize embryos that have been injured in the process in 200 µg/mL Tricaine solution.

3.2. Preparation of IAV for microinjection

Note: Strains that have been shown to infect zebrafish embryos are influenza A/PR/8/34 (H1N1) (APR8), influenza A X-31 A/Aichi/68 (H3N2) (X-31), and the fluorescent reporter influenza strain NS1-GFP³³. Details about APR8 and X-31, and other strains, can be found at the Influenza Research Database (www.fludb.org).

3.2.1. Propagate the NS1-GFP strain of IAV in embryonated chicken eggs as previously described^{34,35}. Collect, aliquot, and titer the allantoic fluid containing IAV, and store at -80 °C. Pre-titered APR8 and X-31 viruses can be purchased commercially.

3.2.1.1. Just prior to infection, rapidly thaw an IAV aliquot in gloved hands and then quickly place on ice to reduce loss of titer.

3.2.2. Viral dilution

3.2.2.1. If using the APR8 or X-31 viruses, dilute to 3.3×10^6 egg infective dose 50%

(EID50)/mL in sterile, ice-cold PBS supplemented with 0.25% phenol red (to aid in visualization) in a laminar flow hood. Simultaneously set up a control containing sterile PBS with 0.25% phenol red.

3.2.2.2. If using NS1-GFP strain³³, dilute to $\sim 1.5 \times 10^2$ plaque forming units (PFU) per nL in sterile, ice-cold PBS containing 0.25% phenol red (to aid in visualization) in a laminar flow hood. Simultaneously set up a control containing allantoic fluid from uninfected chicken eggs diluted like the virus in sterile PBS with 0.25% phenol red.

3.2.2.3. Maintain IAV on ice until ready to use.

3.2.3. Pipet virus solution into microinjection needles using microloader tips just prior to use. Insert microinjection needle into the appropriate holder of the injection apparatus (e.g. MPPI-3 pressure injection system).

3.2.4. While viewing under the stereo microscope, gently clip the microinjection needle tip with sharp, sterilized #5 forceps.

NOTE: It is recommended that the microinjection tip be clipped gradually.

3.2.5. Depress the foot pedal of the pressure injection system and inject into a drop of microscope immersion oil on a calibration micrometer slide. Measure the diameter of the drop. Calculate the volume of the drop using the equation, $V=4/3\pi r^3$, where V is volume in nL and r is the radius in microns.

NOTE: If the drop diameter is too small, additional glass may be clipped or pressure and timing settings can be adjusted. If the drop diameter is too large, pressure and timing settings can be adjusted.

3.2.6. Adjust pressure and timing settings on the pressure injector and/or reclip the needle tip until the desired injection volume is achieved (typically 1-3 nL). Pressure settings between 20 and 30 psi and pulse duration settings between 40 and 80 msec are typical. Adjust back pressure so that it counters capillary pressure but does not leak IAV (net zero pressure).

3.3. Align embryos for injection

3.3.1. Anesthetize dechorionated embryos in 200 μ g/mL Tricaine.

3.3.2. Once movement ceases, transfer 10-20 embryos to a 2% agarose plate (prepared in section 2.3) with a plastic pipette. Use plastic pipette to remove excess liquid.

3.3.3. Gently align embryos for microinjection with a fire-polished and sealed borosilicate glass capillary. Using a stereo microscope, gently orient embryos so that the duct of Cuvier or the posterior cardinal vein is in line with the microinjection needle (Figure 1A).

3.4. IAV microinjection

3.4.1. Gently insert the microinjection needle into the duct of Cuvier or the posterior cardinal vein. Depress foot pedal to inject the desired volume of IAV into the circulatory system of the embryo (Figure 1A). Embryos can be injected more than one time if necessary.

Note: The injection bolus should be swept up into the circulation and distributed throughout the body. If injection volume collects at the site of injection, remove embryo from experiment and euthanize.

3.4.2. Repeat injections on different embryos with control solution specific to the strain of virus chosen. For APR8 and X-31, use the control described in section 3.2.2.1; for the NS1-GFP, use the control described in section 3.2.2.2.

3.5. Transfer embryos to labeled Petri dishes containing sterile egg water and place in incubators at 33°C.

NOTE: Infected embryos should be grown at 33 °C to support enhanced viral replication. In addition, incubation at 33 °C more closely mimics the temperature of the human upper respiratory tract, which is in close contact with lower temperatures in the external environment and is where IAV infections occur. Embryos can acclimate to a broad temperature range ³⁴.

4. Localized, swim bladder IAV infection in *Tg(mpx:mCherry)* transgenic zebrafish (5 d post-fertilization)

4.1. Preparation of IAV for microinjection

4.1.1. Dilute the NS1-GFP strain and control injection solution as described in section 3.2.

4.1.2. Prepare needles as described in section 2.2.

4.1.3. Align *Tg(mpx:mCherry)*³⁵ larvae containing inflated swim bladders as described in section 3.3 with the following exception. Align larvae in such a way that the needle can pierce the swim bladder and the virus can be deposited into the posterior of the swim bladder, as previously described³⁶ (Figure 2A).

4.2. IAV microinjection

4.2.1. Gently insert the microinjection needle into the swim bladder and inject the desired volume (e.g. 5 nL) towards the posterior of the structure (Figure 2A).

Note: The injection bolus should collect toward the posterior and the swim bladder's air bubble should be displaced forward. GFP expression should begin to be observed at 3 h post-injection.

4.2.2. Repeat injections with control solution specific to the strain of virus chosen, as described in section 3.4.2.

4.3. Transfer larvae to Petri dishes containing sterile egg water and place in incubators at 33

°C.

5. Antiviral drug treatment

Note: The protocol below describes the Zanamivir treatment previously shown in Gabor *et al.*¹⁹. This protocol can be modified to screen other antiviral drugs and has the potential to be modified to screen multiple compounds in a 96 well plate, high-throughput format.

5.1. At 3 h post infection, replace egg water of NS1-GFP- and control-infected fish with sterile egg water containing 0, 16.7, or 33.3 ng/mL Zanamivir.

Note: These concentrations were chosen in order to attempt to replicate the physiological levels achieved following administration of Zanamivir in human patients (100, 200, or 600 mg, i.v., twice daily or 10 mg inhaled, twice daily). The serum concentrations in human patients ranged from 9.83 to 45.3 ng/mL³⁶.

5.2. Over the course of 5 days, change egg water every 12 h and replace with sterile egg water containing the appropriate amount of Zanamivir.

5.3. Track course of infection.

5.3.1. Anesthetize zebrafish in 200 µg/mL Tricaine. Monitor GFP expression by stereo fluorescence microscopy to visually observe differences in infection patterns between IAV-infected and control fish and among the fish immersed in the different concentrations of drugs.

5.3.2. Track morbidity and mortality over the course of 5 days.

5.3.2.1. Record observations about infection dynamics related to disease pathology, including signs of lethargy and evidence of edema, differences in pigmentation, ocular and craniofacial deformities, and lordosis. Disease pathology typically becomes apparent in control infected fish at 24-48 h post-injection, depending on the amount of virus injected. Collect images 24 h post-injection.

5.3.2.2. Every 24 hours for 5 days post-infection, record the number of dead, morbid, and healthy larvae. Death is defined by the absence of a discernible heartbeat. Plot data as desired. For example, plot data as a stacked bar graph, with percentages of healthy, morbid, or dead fish plotted on the y-axis and the treatment group on the x-axis.¹⁹

Note: Mortalities may be scored by Kaplan-Meier survival estimates. Depending on application, it may be appropriate to develop alternative approaches for scoring fish morbidity, including a scoring matrix that may quantify the degrees of morbidity based on the aforementioned pathological features observed.

5.3.2.3. Consult with a statistician to apply the appropriate statistical analyses.

6. Neutrophil migration

Note: The protocol below describes a method for tracking neutrophil migration to the swim bladder following a localized, epithelial IAV infection. The methods described can be modified to test the effects of genetic and chemical manipulations. Using this technique, it will be possible to characterize the mechanisms underlying neutrophil behavior during an IAV infection.

6.1. Mounting zebrafish for imaging

6.1.1. Anesthetize larvae to be imaged in 200 µg/mL Tricaine.

6.1.2. Transfer an individual *Tg(mpx:mCherry)* larva that has been infected with NS1-GFP to a well of a 24 well, glass bottom plate in a small droplet of egg water (~50-100 µL). Repeat with other IAV-infected and control fish.

6.1.3. Slowly add 1% agarose embryo mounting media to each well (section 2.5), taking care not to introduce large bubbles.

6.1.3.1. Gently adjust the position of the larva so that it is mounted on its side. Goody and Henry³⁷ describe how to make probes that function well in this application using insect pins that are mounted in borosilicate glass capillaries with filaments and superglued in place.

6.1.4. Once the agarose hardens, gently fill the individual wells with egg water containing 200 µg/mL Tricaine.

6.1.5. With a confocal microscope, capture a z stack series of brightfield and fluorescence images using a 20x objective focused on the swim bladder.

6.1.5.1. Set the upper and lower limits so that they capture all visibly observable green and red fluorescence.

6.1.5.2. Capture images at 2.0 µs/pixel with steps that are ≤ 4 µm. Increasing time per pixel and reducing distance between steps (e.g. 0.5 – 1.0 µm) increases picture resolution and quality.

6.1.5.3. Generate a two-dimensional image by merging layers.

6.1.5.4. Compare the number of mpx-mCherry-positive neutrophils present in the swim bladders of IAV-infected and control-injected zebrafish.

REPRESENTATIVE RESULTS:

Here, data showing how systemic IAV infection in zebrafish can be used to test drug efficacy (Figure 1A) are provided. Embryos at 48 h post-fertilization are injected with APR8 (Figures 1C, 1F), X-31 (Figures 1D, 1G), or NS1-GFP (Figures 1H-1I) via the duct of Cuvier to initiate a viral infection. Another cohort of embryos at 48 h post-fertilization were injected to serve as

controls for viral infection (Figures 1B, 1E). By 48 h post-infection, zebrafish injected with IAV exhibited evidence of pericardial edema (Figures 1C, 1D) and circulatory arrest (Figure 1F), with erythrocytes present throughout the pericardium (Figure 1G). Using NS1-GFP, the initial expression of GFP by fluorescence microscopy as early as 3 h post-infection was observed. At that point, zebrafish were exposed to a 33.3 ng/mL dose of the neuraminidase inhibitor Zanamivir. This dose is roughly equivalent to the 200 mg dose given to human patients. Control infected fish not exposed to Zanamivir (Figures 1H, 1H') exhibited features of edema and systemic GFP expression. Reduced gross pathology and GFP fluorescence (Figures 1I, 1I') in NS1-GFP-infected larvae exposed to Zanamivir were observed. The reduction in GFP was most noticeable in the bodies of larvae, while very little change was observed in the yolk sacs. Fish treated with Zanamivir exhibited less morbidity, as evidenced by improved swimming motility and reduced levels of edema in the heart, and improved survival. These findings complement a more comprehensive study¹⁹ and show the value of this model for drug screening.

A localized zebrafish swim bladder infection model³¹ has been adapted for IAV infection. NS1-GFP virus was injected into the swim bladders of 5 d post-fertilization *Tg(mpx:mCherry)* larvae to initiate a localized, epithelial infection (Figure 2A). PBS was injected into the swim bladder as a control to demonstrate the specificity of neutrophil recruitment toward IAV-infected cells. The recruitment of mCherry-labeled neutrophils into the swim bladder was tracked. At 20 h post-infection, considerable migration of neutrophils into the swim bladders of IAV-infected fish relative to PBS-injected controls (Figures 2B, 2B', 2C, 2C') was observed. These data demonstrate that IAV can recruit neutrophils to the swim bladder, just as it recruits neutrophils to the human lung.

Figure 1: Antiviral drug treatment mitigates the severity of IAV infection in zebrafish.

(A) Schematic demonstrating possible IAV injection sites to initiate systemic infections in a 48 h post-fertilization embryo. Embryos can be injected at multiple sites in the vasculature to initiate a systemic infection, including the duct of Cuvier and the posterior cardinal vein just above the yolk extension. (B-I) Gross pathology and GFP fluorescence of zebrafish embryos injected into the duct of Cuvier with IAV. (B-G) Fish infected with IAV and examined at 48 h post-infection for signs of viral infection and disease. (B-D) Single focal planes of control or IAV-infected fish were collected (side-mounted, anterior left, dorsal top, $\times 4$ magnification, scale bar = 500 μm). (B) Control fish were injected with PBS and display normal morphology. (C-D) Embryos infected with APR8 (C) or X-31 (D) commonly exhibited pericardial edema (filled arrow). Embryos also had necrotic tissue, evident in (C). (E) Control fish were injected with PBS and display no evidence of pathology (scale bar = 250 μm) (F) Embryos infected with APR8 displays circulatory arrest (notched arrowhead, scale bar = 250 μm). (G) Embryo injected with X-31 influenza displays both pericardial edema (arrow) and pooled erythrocytes throughout the pericardium (notched arrowhead, scale bar = 100 μm). (H,I) Representative images showing effects of an antiviral drug on IAV-infected zebrafish (scale bar = 200 μm). (H) Brightfield and (H') fluorescence image showing an embryo injected with NS1-GFP (no antiviral drug treatment). Notice edema and GFP expression especially in the common cardinal vein region. (I, I') Treatment with an antiviral drug reduced the infection, as evidenced by reduced pathology, including diminished edema, and diminished GFP expression within the white dashed outline of

the body, particularly in the vasculature, which is indicative of reduced viral burden.

Figure 2: Neutrophils are recruited to sites of localized IAV infection.

(A) Schematic demonstrating the injection approach needed to initiate a localized IAV infection in an inflated zebrafish swim bladder at 5 d post-fertilization. (B, C) Neutrophil recruitment to the swim bladder following NS1-GFP infection. Z-stack images (4 μ m steps) were collected by confocal microscopy ($\times 20$ magnification). Images were flattened into two-dimensions (side-mounted, anterior left, dorsal top). *Tg(mpx:mCherry)* zebrafish (5 d post-fertilization) were injected with (B,B') allantoic fluid diluted with PBS and 0.25% phenol red or (C,C') NS1-GFP (7.2×10^2 PFU/embryo) diluted in allantoic fluid and PBS with 0.25% phenol red. At 16 h post-infection, neutrophils were present in an IAV-infected swim bladder (C, C': green fluorescence shows localized infection; C': red cells are neutrophils, white arrow identifies representative neutrophil). (B, B') No evidence of GFP expression indicative of IAV infection and no recruitment of neutrophils to the swim bladder was observed in the larvae injected with the allantoic fluid in PBS.

DISCUSSION:

To maximize the benefits gained from using a small animal to model human host-pathogen interactions, it is important to frame research questions and test hypotheses that capitalize on the inherent advantages of the model system. As a model for human IAV infection, the zebrafish has several strengths, including high fecundity, optical clarity, amenability to drug screening, and availability of transgenic lines that label immune cells like neutrophils. The zebrafish has been developed as an increasingly powerful alternative to the mouse model system for the study of inflammation and innate immunity. Because they lack a functioning adaptive immune response during the first 4-6 weeks of development, zebrafish mount innate immune responses to protect against injury and infection³⁷. During this early period in development, it is possible to isolate and study mechanisms of the antiviral response arising from the innate immune response alone. This work has been facilitated by the development of transgenic zebrafish lines like *Tg(mpx:mCherry)*, which labels neutrophils with a red fluorescent protein.

Zebrafish models for IAV infection that resemble human disease were recently described¹⁹. It was demonstrated that zebrafish possess α -2,6-linked sialic acid residues on their cells that provide IAV viruses the tropism to bind, attach, and enter cells. In this manuscript and in Gabor et al.¹⁹, it has been shown that two different strains of IAV (A/PR/8/34 [H1N1] and X-31 A/Aichi/68 [H3N2]), as well as the recombinant NS1-GFP strain that results in GFP translation in infected cells, could infect, replicate, and cause mortality when injected into the circulatory system of larval zebrafish. Injection of the virus is critical to this infection method, as exposure to IAV via immersion does not work reliably. Infected fish should be transferred to a 33 °C incubator to ensure viral replication. It is important to note that the human respiratory tract, where influenza infections occur, is typically 33 °C. Incubation at 28 °C, which is more typical temperature for zebrafish incubation, fails to produce a reliable infection. Further, it is essential to test each lot received from the manufacturer or batch of IAV produced prior to use in an experiment due to variability that affects infectivity and disease progression in the zebrafish.

When the infective dose is titrated correctly, the vast majority of zebrafish infected with these strains of IAV should exhibit gross disease phenotypes typified by edema, as early as 24 h post-infection, that progressively worsen, craniofacial abnormalities by 48 h post-infection, lordosis by 72 h post-infection, and approximately 50% should succumb to the infection by 5 d post-infection. Histopathology at 48 h post-infection should include evidence of gill, head kidney, and liver necrosis, as well as indications of fluid in the pericardium. In establishing these findings as the predictable outcome of an IAV infection in zebrafish, it is possible to screen candidate anti-influenza drug compound candidates. With this in mind, a proof-of-principle protocol for screening an antiviral drug based on original findings described in Gabor et al.¹⁹ are demonstrated. Using the neuraminidase inhibitor Zanamivir, at a dose that is predicted to mimic levels observed in human blood, delayed onset of morbidity and reduced mortality through an apparent effect on viral replication/spread, as determined by NS1-GFP fluorescence, was observed. The zebrafish IAV model is ideally suited for moderate- to high-throughput small molecule screens. Because infections can only occur only through manual injection into the circulation via the duct of Cuvier or posterior cardinal vein, the size of the screens are limited by the number of technicians establishing the infections and their proficiencies in performing the technique. Nevertheless, it is possible to inject at least 200 zebrafish embryos per technician per hour. While successful in demonstrating antiviral activity for Zanamivir in a zebrafish infection model, it must be acknowledged that drug screening in all animal models, including zebrafish, must be carefully controlled³⁸. The way a drug is absorbed and metabolized differs from animal-to-animal, and is difficult to predict. Nevertheless, as a method for screening new anti-influenza drugs, this zebrafish IAV infection model presents an exciting opportunity to survey many thousands of small molecules in an animal with organs orthologous to humans and with highly-conserved integrative physiology.

In addition to being a model for systemic infection, the zebrafish can also serve as a model for localized infection when IAV is injected into the swim bladder¹⁹, the functional analogue of the human lung^{21,29-31}. When properly titrated, zebrafish that are infected with the NS1-GFP strain at 5 d post-fertilization should exhibit evidence of punctate fluorescence around the epithelia of the swim bladder by 1 d post-infection. Using this localized infection strategy, neutrophil behavior in response to the infection can be tracked. Like human neutrophils, zebrafish neutrophils are a principal cellular mediator in innate immunity, functioning during acute responses to tissue injury and infection and playing critical roles during chronic inflammation²⁴. There is a growing recognition that neutrophils play a crucial role in the immune response to influenza infection. Indeed, it has become apparent that neutrophils function as “double-edged swords” in mediating the immune response. While essential to the antiviral response necessary to control influenza infections, neutrophils also contribute to an excessively inflammatory environment in the lung that can damage host tissues and increase the risk of mortality³⁹⁻⁴³. There is considerable conservation of gene synteny between the zebrafish and human genomes, and with this orthology, there is also significant functional conservation in neutrophil biology. Zebrafish neutrophils bear many similarities to human neutrophils, including a polymorphic nucleus, the presence of primary and secondary granules, and functional myeloperoxidase and NADPH oxidase²². In addition, zebrafish neutrophils can engulf bacteria and release neutrophil extracellular traps (NETs)⁴⁴. Several transgenic zebrafish lines have been

developed to help identify and dissect the functions of neutrophil biology^{27,45-47}. This infection protocol is flexible and can be modified to test multiple neutrophil functions using these alternative tools. This localized zebrafish IAV infection model enables questions related to the host immune response to be addressed, and in particular those mediated by neutrophils.

Studies carried out in mammalian model species have yielded valuable information^{40,48-55}, but few real-time experiments involving IAV infection have been performed, limiting the understanding of the complex interplay among the components of the vertebrate innate immune response in the host. Over the last decade, the use of the zebrafish animal model system has yielded a watershed of information regarding host-pathogen interactions^{21,47,56,57}, and has also offered important information as a tool for drug discovery. A combination of molecular techniques and imaging microscopy have allowed a better understanding of innate immune responses and how these are manifested *in vivo*^{17,18,27}. The discovery that zebrafish can be infected with IAV¹⁹, and that the zebrafish shares important immunological similarities with mammalian systems, including human, has opened the door to the study of an important human viral pathogen. The protocols described are adaptable to other applications and have the potential to yield critical discoveries regarding host-virus interactions that may have profound clinical impacts on human health.

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The authors have nothing to disclose.

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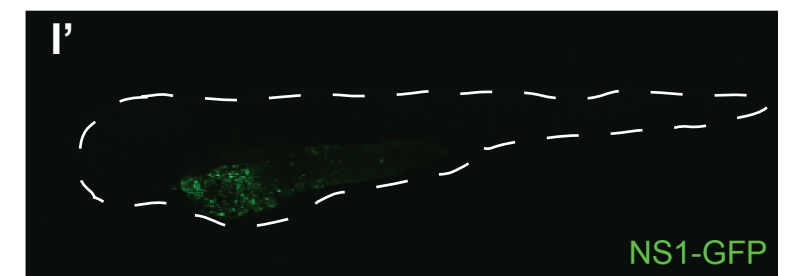
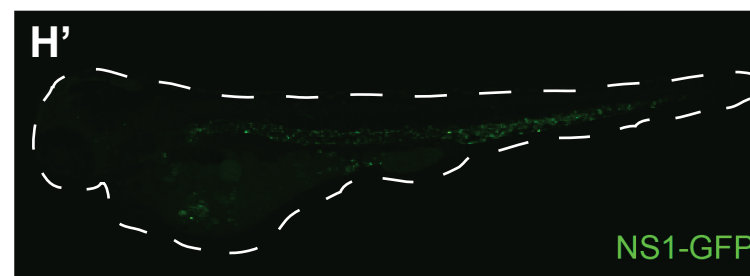
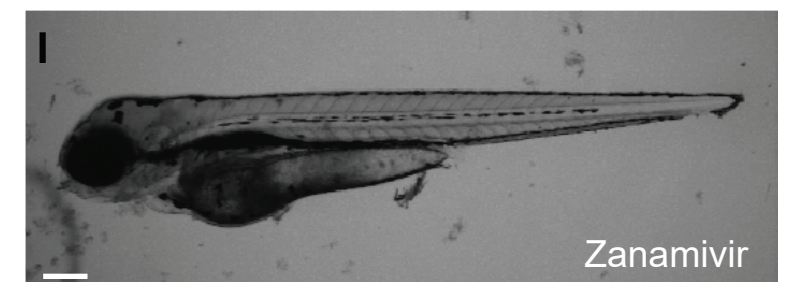
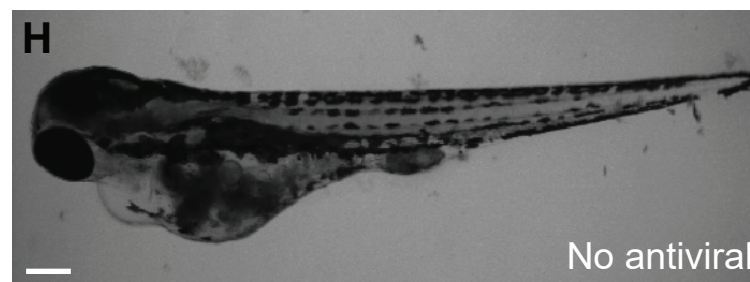
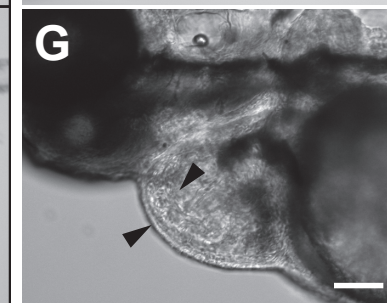
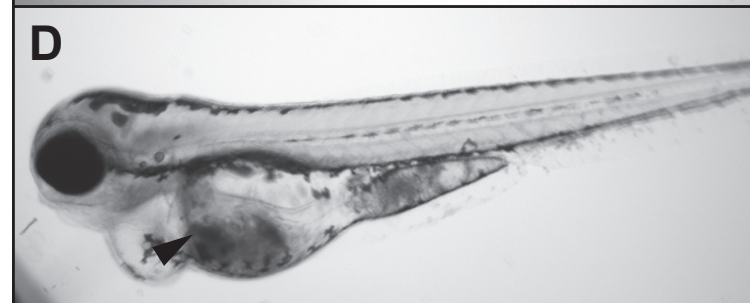
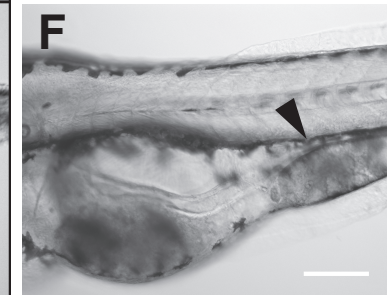
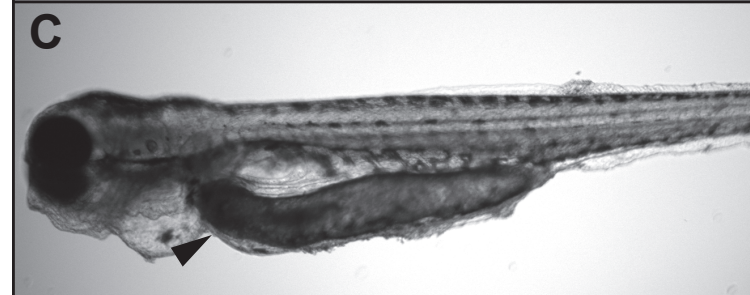
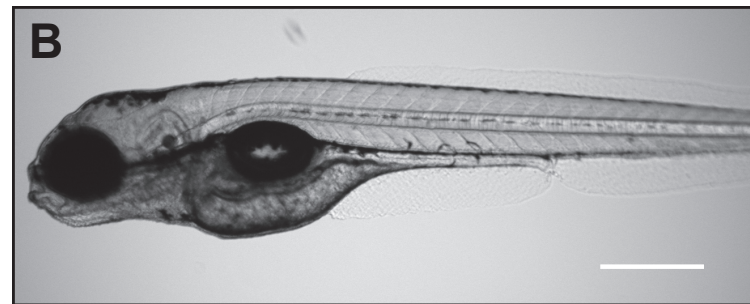
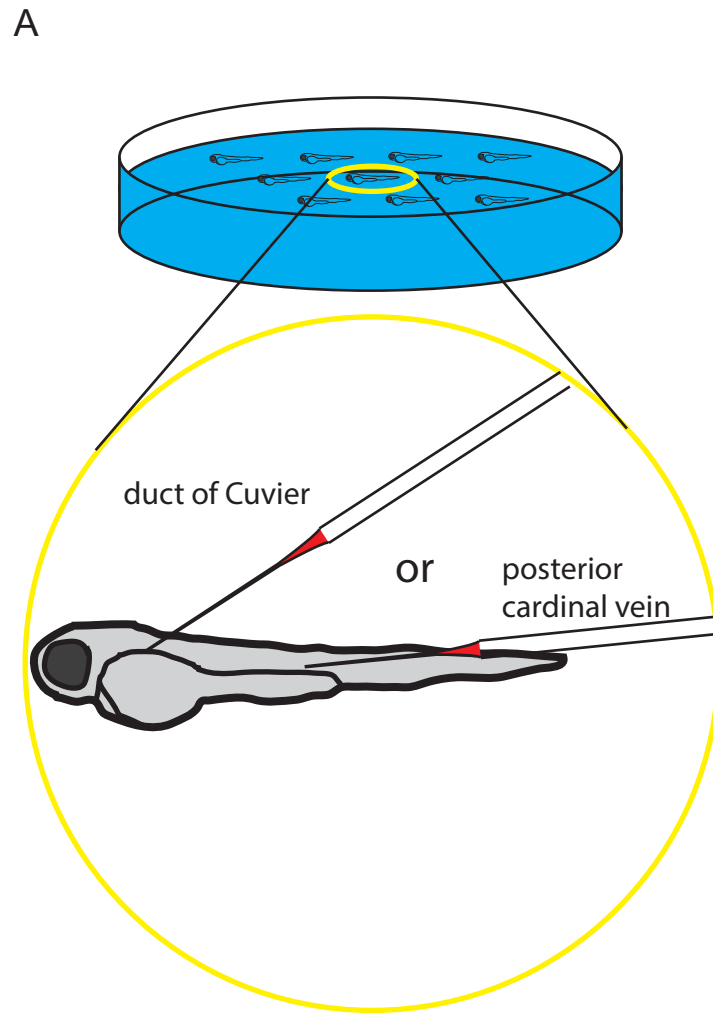
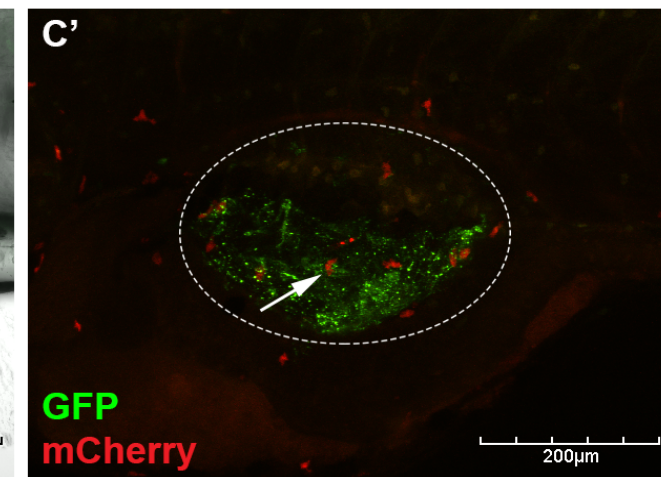
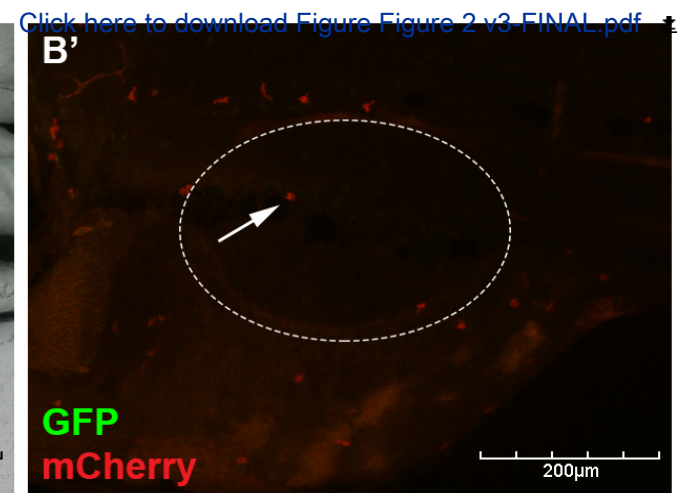
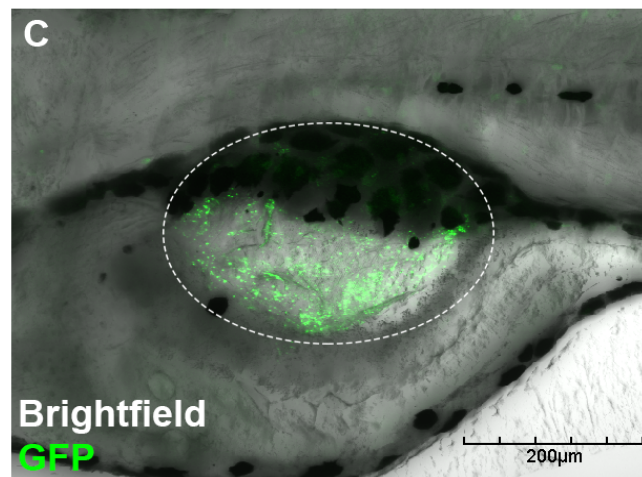
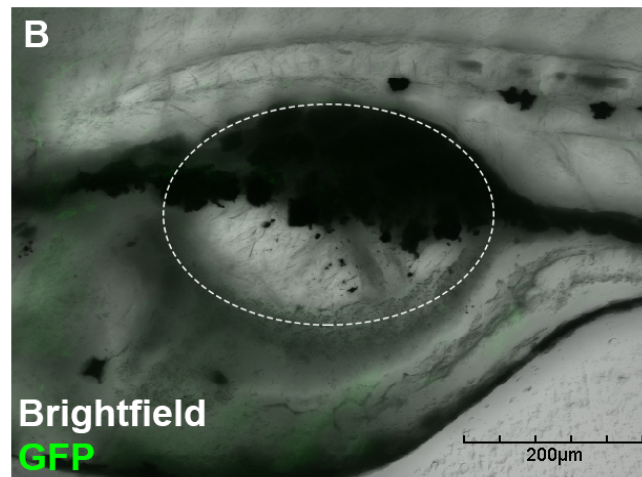
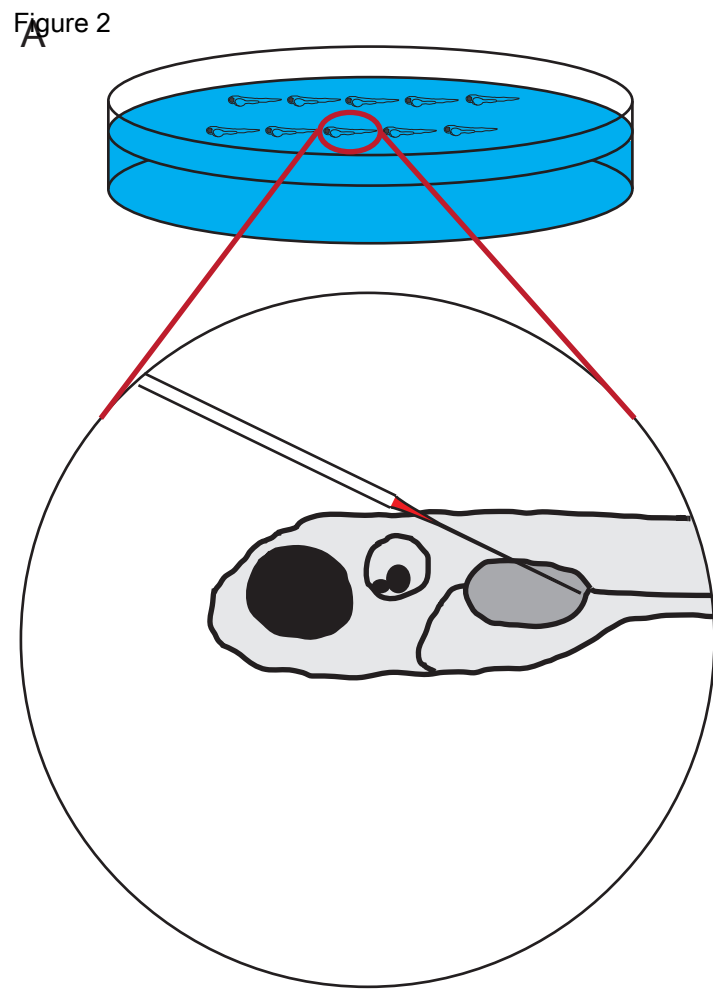


Figure 2



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Instant Ocean	Spectrum Brands	SS15-10	
100 x 25 mm sterile disposable Petri dishes	VWR	89107-632	
Transfer pipettes	Fisherbrand	13-711-7M	
Tricaine- S (MS-222)	Western Chemical		
Borosilicate glass capillary with filament	Sutter Instrument	BF120-69-10	
Flaming/Brown micropipette puller	Sutter Instrument	P-97	
Agarose	Lonza	50004	
Zanamivir	AK Scientific	G939	
Dumont #5 forceps	Electron Microscopy Sciences	72700-D	
Microloader tips	Eppendorf	930001007	
Microscope immersion oil	Olympus	IMMOIL-F30CC	
Microscope stage calibration slide	AmScope	MR095	
MPPI-3 pressure injector	Applied Scientific Instrumentation		
Stereo microscope	Olympus	SZ61	
Back pressure unit	Applied Scientific Instrumentation	BPU	
Micropipette holder kit	Applied Scientific Instrumentation	MPIP	
Foot switch	Applied Scientific Instrumentation	FSW	
Micromanipulator	Applied Scientific Instrumentation	MM33	
Magnetic base	Applied Scientific Instrumentation	Magnetic Base	
Phenol red	Sigma-Aldrich	P-4758	
Low temperature incubator	VWR	2020	
SteREO Discovery.V12	Zeiss		
Illuminator	Zeiss	HXP 200C	
Cold light source	Zeiss	CL6000 LED	
Glass-bottom multiwell plate, 24 well	Mattek	P24G-0-13-F	
Confocal microscope	Olympus	IX-81 with FV-1000 laser scanning confocal system	
Fluoview software	Olympus		
Prism v6	GraphPad		
Influenza A/PR/8/34 (H1N1) virus	Charles River	490710	
Influenza A X-31, A/Aichi/68 (H3N2)	Charles River	490715	
Influenza NS1-GFP			Referenced in Manicassamy et al. 2010
Tg(mpx:mCherry)			Referenced in Lam et al. 2013



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Article Title:

Using zebrafish models of human influenza A virus infections to screen antiviral drugs and characterize host immune cell responses

Signature:



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9/19/16

Dear Dr. Nguyen:

We wish to thank you and the reviewers for the helpful critiques of our manuscript. Below please find a point-by-point rebuttal to critiques (in italics). Modifications to the manuscript have been made in red text.

We thank you for this opportunity and look forward to filming our protocol. Please do not hesitate to contact us with any additional questions or concerns that need to be addressed.

Sincerely yours,

Carol Kim

Editorial comments:

The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (55235_R0_072216.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We thank the editors for the opportunity to proofread our manuscript again.

2. Please abbreviate all journal titles.

We downloaded the current JOVE Endnote style and updated the references to conform to the Endnote style requirement. We then manually changed several journal listings to their abbreviations.

3. Please define all abbreviations before use.

We have identified abbreviations in the text and have defined them.

4. Please print and sign the attached Author License Agreement (ALA). Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.

We will fax the agreement to your office.

5. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have identified personal pronouns within the text and have altered the sentence structures to avoid their use.

6. Additional detail is required: 4.2.2 – presumably the controls are carried out on different fish?

We have addressed this critique by explicitly stating that the control injections are performed on different fish and referring the readers to a modified section 3.4.2, which described the way the control solution should be made.

7. Results: Please add scale bars to Figure 1 images

We have added scale bar to our images in Figure 1.

8. Discussion: Discussion reads more like a typical research paper. Please explicitly discuss the critical steps and limitations of this method and any modifications/troubleshooting that can be performed. Please also address alternative methods when discussing significance.

We have added text at lines 471-496, 504-507, 521-523, and 536-538 to address the concerns raised.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This article describes a method for influenza virus infections in zebrafish larvae and an example of antiviral drug screening. It builds on a previous article with more results but less detailed description of the method. The protocol is well described and easy to follow. Although the results are convincing there are a few points that need to be discussed since this is a methods article:

1. Reproducibility - quantification of infection burden based on imaging should be accompanied with some numbers on reproducibility

In our experience, we have observed penetrance of the described phenotypes in the majority of fish infected with IAV. We have added commentary about this at lines 478-481.

2. HTS potential - for drug screening, injection of virus will put some restrictions on the size of libraries that can be screened

The high throughput screening potential is substantial. We can routinely inject at least 96 embryos within 30 minutes. This has the potential to allow for over 200 compounds to be screened in a single replicate format per hour of work. We have added commentary about this at lines 490-495.

3. Temperature - transferring larvae from 28 to 33 degrees will initiate thermal stress responses in the fish. This needs to be discussed. Have you tested viral replication at 28 degrees?

In our experience, zebrafish infected with IAV should be grown at 33 degrees in order to observe the penetrance of phenotypes we observe. While thermal stress is a consideration, it is important to note that zebrafish can tolerate temperatures in excess of 33 degrees in the wild and that within the experiment, control animals are also injected and maintained at 33 degrees. These concerns are addressed within the manuscript at lines 473-476.

Major Concerns:

N/A

Minor Concerns:

N/A

Additional Comments to Authors:

N/A

Reviewer #2:

Manuscript Summary:

Kim et al describe methods for initiating and monitoring both localized and systemic influenza A virus infection in zebrafish embryos. Protocols for imaging the infected fish and for testing drugs and other small molecules are outlined. The local infection model involves virus injection into the swim bladder, which the authors state is analogous to the lung. The systemic model is initiated by virus injection into the circulatory system via the duct of Cuvier or the posterior cardinal vein. The authors include several protocols, from fish spawning to microscopy following injection. The paper is well written and should be useful for labs wishing to try this or similar infection models. Some additional details concerning some of the approaches would enhance user friendliness and clarity.

Specific Comments:

For the Protocol sections:

1.2 Is there an upper or lower limit for the age of the fish for use in the described work? Is there a point at which the injections become too difficult or imaging the fish is less effective as transparency is lost. Noting some recommended ages for the infections would be helpful.

To address this concern, we provided the developmental ages required in our protocols for systemic and localized infection in the text at section 1.2 and then included a reference to the section each of those protocols can be found. For systemic infection, the lower age limit is point at which substantial blood flow is occurring, typically after 28 h post-fertilization. For the swim bladder infection, the lower age limit is tied to the development of the swim bladder; the upper age limit has not been tested yet.

2.1 On the Directions for Use file, the protocol states that Tricaine-S should not be dissolved in distilled water and does not indicate that Tricaine-S should be autoclaved. This is in contrast to manufacturer's suggestions. Please clarify.

We examined the directions for use file provided by Western Chemical and noticed the text that has caused this concern. As a laboratory, we routinely dissolve our Tricaine S in distilled water and autoclave with no ill effects. We also adjust the pH of our Tricaine S to 7.0-7.4. Our tricaine S stock is then added to egg water (containing Instant Ocean salts) with embryos for anesthesia. We have modified section 2.1 to include additional information.

2.2 Every filament in a micropipette puller is going to have different settings, so it is not clear if this information is helpful. Would suggesting a length from where the needle begins to taper to the end of the needle would be a better option?

Micropipette preferences vary from lab-to-lab. In our experience, we prefer a taper that is gradual and that retains enough strength to pierce zebrafish injection sites without bending and breaking. Prior to

trimming the needle, the length from where the needle begins to taper to the tip of the needle is approximately 12-15 mm.

A note has been added to section 2.2 to address this concern.

2.5 Would be useful to indicate that this will be used later to mount the fish.

A reference to the fact that this will be used as embryo mounting agarose has been added to section 2.5.

3.2 The different influenza strains should be cited and explained better for non-aficionados, not just the NS1-GFP strain.

It was noted that details about APR8 and X-31, and other strains, can be found at the Influenza Research Database (www.fludb.org) in section 3.2.

3.2.1 It isn't clear here if this is how all of the viruses are prepared or just the NS1-GFP strain.

A statement was added to indicate that pre-titered APR8 and X-31 strains can be purchased from Charles River Laboratories.

3.2.2.1 Define out that EID50/mL.

This definition has been added to section 3.2.2.1.

3.2.4 This should be a clearer description about how much of the needle should be trimmed before measuring the diameter of the drop to ensure that the needle doesn't get trimmed too far back.

Notes have been added to section 3.2.4 and 3.2.5 to provide further guidance regarding needle preparation.

3.3.2 Would help to state that this 2% agarose plate was prepared earlier in the protocol

Parenthetical text has been added to section to indicate how plates were prepared in section 2.3.

3.4.2 Should make it clearer that there are two separate control solutions.

The text was clarified to indicate when each of the control solutions should be applied.

3.5 Why are the embryos now stored at 33°C instead of 28°C?

The higher temperature facilitates IAV replication. This has been noted and a new reference has been added to section 3.5. It is described further in the discussion section at lines 478-481.

4.2.2 Again, should make it clear that there are two separate control solutions.

The text was modified to clarify this section. A reference to section 3.4.2. was also provided.

5.3 Additional details for monitoring course of infection would aid the reader and would-be experimenter. Can the authors suggest time intervals for imaging the fish? How is death defined? Lack of heart beat, lack of blood flow?

The text has amended in section 5.3.2.1 to describe that disease pathology typically becomes apparent in control infected fish at 24-48 h post-injection, depending on the amount of virus injected, and that it is recommended that images begin to be collected 24 h post-injection. A sentence that death is defined as the absence of a discernible heart beat has been added to section 5.3.2.2.

5.3.1 It would be helpful to provide some more detail about how to monitor GFP expression, as trying to do it in the dish where the fish are free to swim around can be difficult

A sentence indicating that zebrafish should be anesthetized in tricaine has been added.

5.3.2.3 It would be helpful if the authors provided some examples of the types of stats that have been used appropriately to assess death rates and morbidity. It seems that having blinded observers could be helpful when scoring morbidity. Is there a scoring matrix that can be used to better quantify levels (degrees) of morbidity in zebrafish?

The suggestions offered by the reviewer are appreciated, and we have offered a note in section 5.3.2.2 to address this concern. We also reference our manuscript in DMM to offer potential methods by which death rates and morbidities can be scored. It is also appropriate to perform Kaplan-Meier survival estimates, and we added a sentence to the note to include this fact.

- It is surprising that there is no protocol for enumerating virus titers present in the zebrafish? This seems like it would be a very useful inclusion.

We excluded enumerating virus titers from this protocol because it is intended for use as a high throughput screen. We respectfully would argue that the calculation of viral titers in this application is not compatible with a high-throughput screen. We were invited and have submitted a book chapter to Methods in Cell Biology that will describe viral titer protocol for other applications.

Representative Results section:

- At the end of the first paragraph a reference to Figure 1F should be made: "By 48 h post-infection, zebrafish injected with IAV exhibited evidence of pericardial edema (Figure 1C,D) and circulatory arrest (Figure 1E), with erythrocytes present throughout the pericardium (Figure 1F)."

We have modified the text to include this correction.

- line 359, it is not clear what control animals are injected with. PBS alone?

We injected the control animals in this case with PBS and address it on line 426 in the revision.

- lines 369 - 372: It would be useful to describe in the protocols how the authors score for "improved swimming behaviours, reduced edema, and reductions in overall mortalities".

We have clarified the sentence to indicate fish treated with Zanamivir exhibited less morbidity, as evidenced by improved swimming motility and reduced levels of edema in the heart, and improved survival (lines 403-404).

- Figure 1: It would be helpful to include on the figure labels in each panel to indicate which IAV was injected. A zoomed in panel of a control injected fish would make the observations in E and F more evident. As it stands now, I have a difficult time knowing what exactly I'm looking at in these panels. In panels G and H it would be helpful to include labels such as no treatment and treated with Zanamivir. Additionally, it would help to indicate the drug used to treat the fish in the figure legend, not just stating that an antiviral drug was used. In panels G and H, arrows indicating edema, with comparisons to a healthy control lack would be helpful. In panels G' and H'. it seems that there is still a lot of GFP expression, but it is more localized to the yolk or nearby regions in drug treated fish. Are virus titers actually reduced with drug treatment, or just spatially shifted? Sites of injection should be indicated in the legend.

We have modified Figure 1 and the Figure 1 legend to include these helpful suggestions. In our observations, we observed a reduction in GFP in the vasculature as a consequence of drug treatment (lines 440-441). We did not observe a perceptible change in fluorescence in the yolk. The site of injection at the duct of Cuvier was noted on line 423.

Table of Materials/Equipment:

- Missing are references/sources for the NSI-GFP Strain and the Tg(mpx:mCherry) Line.

We have corrected the table to include this information.

Major Concerns:

N/A

Minor Concerns:

N/A

Additional Comments to Authors:

N/A

Reviewer #3:

Manuscript Summary:

The manuscript entitled: "using zebrafish models of human influenza virus infections to screen antiviral drugs and characterize host immune cell responses" by Kim et al. develop two different protocols of zebrafish larvae viral infection (particularly influenza A virus). This model infections could be use to screen antiviral drugs such as Zanamivir.

Major Concerns:

N/A

Minor Concerns:

In section 3.3 (line 237) the authors indicate: "align the embryos...". Should they be align in any kind of mold/in-agarose mold? Please specify.

We appreciate the need for clarification on this point as it was also mentioned by reviewer #2. We have added text to section 3.3.2 that we hope addresses this concern.

In section 5 they described how to perform the drug treatment with Zanamavir. They reference the work by Gabor et al. where the Zanamivir is diluted in sterile water and replaced every 12 h. In this reference I did not find the half-life of the antiviral compound but since they replaced the water every 12 h the half life should be maximum 12 h. I think that should be a good idea to include the Zanamavir half life and also would be nice to indicate the importance of taking into account the half-life of any drug in zebrafish water and the solubility as well.

The zanamivir serum half-life is 2.5 -5.1 hours. We added a note to section 5.1. that the concentrations were chosen in order to attempt to replicate the physiological levels achieved following administration of zanamivir in human patients (100, 200, or 600 mg, i.v., twice daily or 10 mg inhaled, twice daily). The serum concentrations in human patients ranged from 9.83 to 45.3 ng/mL.

In section 5.3.2.2 Why do not the authors indicate only to plot the results as a bar graphs? Why do not use survival curves?

In our original publication in DMM, we presented our data as described in this section. It is also appropriate to perform Kaplan-Meier survival estimates, and we added a sentence to include this fact to this section as part of a note in section 5.3.2.2.

Additional Comments to Authors:

N/A