**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. Altghough 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 resrtictions 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 in 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 to difficult or imaging the fish is less effective as transparency is loss. 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*](http://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 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 charachterize 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