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TITLE:

Infection of Zebrafish Larvae with *Aspergillus* Spores for Analysis of Host-Pathogen Interactions

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KEYWORDS:

zebrafish, *Aspergillus*, microinjection, hindbrain ventricle, invasive aspergillosis, immune response, macrophages, neutrophils, confocal imaging, transgenic

SUMMARY:

This protocol describes an *Aspergillus* infection model in zebrafish larvae. *Aspergillus* spores are microinjected into the hindbrain of larvae, and chemical treatment is used to induce immunosuppression. Infection progression is monitored via a daily imaging setup to monitor fungal growth and immune responses as well as enumeration of live spores by colony forming unit plating.

ABSTRACT:

Invasive aspergillosis (IA) is the most common fungal infection among immunocompromised individuals. Despite the availability of antifungal drugs, IA can cause >50% mortality in infected immunocompromised patients. It is crucial to determine both host and pathogen factors that contribute to infection susceptibility and low survival rates in infected patients in order to develop novel therapeutics. Innate immune responses play a pivotal role in recognition and clearance of *Aspergillus* spores, though little is known about the exact cellular and molecular mechanisms. Reliable models are required to investigate detailed mechanistic interactions between the host and pathogen. The optical clarity and genetic tractability of zebrafish larvae make them intriguing models to study host-pathogen interactions of multiple human bacterial and fungal infections in a live and intact host. This protocol describes a larval zebrafish *Aspergillus* infection model. First, *Aspergillus* spores are isolated and injected into the zebrafish hindbrain ventricle via microinjection. Then, chemical inhibitors such as immunosuppressive drugs are added directly to the larval water. Two methods to monitor the infection in injected larvae are described, including the 1) homogenization of larvae for colony forming unit (CFU) enumeration and 2) a repeated, daily live imaging setup. Overall, these techniques can be used to mechanistically analyze the progression of *Aspergillus* infection in vivo and can be applied to different host backgrounds and *Aspergillus* strains to interrogate host-pathogen interactions.

INTRODUCTION:

Aspergillus fumigatus is a ubiquitous saprophytic fungus, and its airborne spores can be found both indoors and outdoors[1]. These spores are inhaled by everyone but become effectively cleared from the lungs of immunocompetent individuals[1,2]. However, people with altered lung conditions such as cystic fibrosis can develop bronchopulmonary aspergillosis due to fungal germination in the lungs[3]. The most severe form of this infection, invasive aspergillosis (IA), affects immunocompromised individuals and involves growth of the fungus into other organs[2,3]. IA leads to >50% death of infected patients despite the availability of anti-fungal therapies[4]. In immunocompetent individuals, innate immune responses play a major role in clearing the inhaled spores[1]. However, the specific mechanisms that contribute to this innate immune clearance are not well-understood. It is important to understand the cellular and molecular mechanisms of major innate immune cells (i.e., macrophages and neutrophils) in clearance of *Aspergillus* in order to find novel therapeutic strategies for IA.

While mammalian models have been instrumental in identifying fungal virulence factors and host immune responses[5,6], visual accessibility is limited for host-pathogen interactions at the cellular level. Tissue culture experiments cannot fully recapitulate the complex multi-cellular environment and interactions that exist in whole animals[7]. Therefore, zebrafish has gained popularity as an alternative model organism to fill this gap and facilitate the study of host-pathogen interactions in a live, intact host across a multi-day infection[8,9]. The zebrafish innate immune system develops as early as 24 h post-fertilization (hpf)[10], and the adaptive system takes 4–6 weeks to develop[11], providing a window of time in which innate immune responses can be assessed in isolation. Innate immune responses are well-conserved between humans and zebrafish[11]. Zebrafish have many qualities that facilitate the investigation of these responses, including optical clarity (which allows for the high-resolution live imaging of intact hosts) and genetic tractability (which facilitates molecular mechanistic studies).

The larval zebrafish *Aspergillus* infection model described here was originally developed by Knox et al.[12]. It has recently been expanded by our group and others to investigate host immune mechanisms[12,13], host-pathogen interactions[13-15], mechanisms of immunosuppression[13,16,17], fungal virulence[18], and anti-fungal drug efficacy[19,20]. This model recapitulates multiple aspects of human aspergillosis. While immunocompetent larvae are resistant, immunocompromised larvae can succumb to infection[12,13,16,17].

In this model, a localized infection is established by injecting spores into the hindbrain ventricle of larva, an area less populated with phagocytes, and phagocyte recruitment and behavior can be evaluated[12,13]. It is believed that macrophages act as the first line of defense against *Aspergillus* spores in humans[1] and mammalian models[6,21]. Similarly, in the zebrafish model, macrophages are recruited to the injected *Aspergillus* spores, while neutrophils are recruited secondarily in response to hyphal growth[12,13,22]. From this model, it has also been learned that *Aspergillus* can persist in wildtype immunocompetent larvae after more than 7 days of infection. Furthermore, the entire course of the infection can be followed in the same live animals by daily confocal imaging.

This protocol describes the technique of microinjection to inject spores into the hindbrain ventricle of 2 days post-fertilization (2 dpf) larvae. The infection is then monitored for up to 7 days, as zebrafish larvae can live up to 10 dpf without feeding. Immunosuppression can be induced by drug treatment, and the application of drugs to the larvae is also described. Finally, two methods to follow infection progression are described, including quantification of CFUs from individual larvae and a daily live imaging setup.

PROTOCOL:

Researchers should obtain approval for all animal experiments from the appropriate animal care and use committees. Representative data shown in this article are from experiments performed under protocols approved by the Clemson University Institutional Animal Care and Use Committee (AUP2018-070, AUP2019-012).

1. Preparation of *Aspergillus* spores for injection

1.1 From an *Aspergillus* spore suspension, calculate the volume needed to obtain 1 x 10^6 spores. The volume should be 20–100 µL; if not, produce a 10x dilution in 0.01% (v/v) sterile Tween-20 (Tween-water; **Table of Materials**). For example, if the calculated volume is 5 µL, produce a 10x dilution and use 50 µL of the diluted solution.

NOTE: Two plates/strain can be prepared to collect more spores or as a spare in the case of contamination.

1.1. Spread 1 x 10^6 *Aspergillus* spores on one glucose minimal media (GMM) plate (**Table of Materials**) with a sterile disposable L-shaped spreader in a biosafety cabinet. Avoid spreading to the margin of the plate. Incubate at 37 °C for 3–4 days, with the plate facing upside down.

1.2. On the day of collection, bring sterile miracloth and 50 mL conical tubes (two per strain), fresh bottles of sterile Tween-water (one per strain), and sterile disposable L-shaped spreaders to the biosafety cabinet.

NOTE: Miracloth can be cut into ~8 in x 6 in pieces, wrapped in foil, and autoclaved to sterilize.

1.3. Place one piece of miracloth in each labeled 50 mL conical tube and re-cap. Take the remaining miracloth packet out of the hood.

1.4. Bring plates into the biosafety cabinet. Open one plate, then pour Tween-water on the top to cover about three-quarters of the plate.

1.5. Using a disposable L-shaped spreader, gently scrape the surface of the fungal culture in a back-and-forth motion, while using the other hand to rotate the plate. Scrape until almost all of the spores are homogenized into the Tween-water.

NOTE: Due to high hydrophobicity, spores can create “puffs” when Tween water is added or during scraping. Great care should be taken to avoid contamination of nearby tubes or plates. It is advised to change gloves and wipe off the surface with 70% ethanol between extraction of different strains.

1.6. Take one 50 mL conical tube and remove the piece of miracloth. Fold it in half and make it into a filter inserted in the top of the 50 mL conical tube.

1.7. Pour the fungal homogenate from the plate over the miracloth into the tube.

NOTE: If two plates of one strain are prepared, scrape both strains and pour them into the same conical tube.

1.8. Pour Tween-water to bring the total volume in the conical tube to 50 mL.

1.9. Spin at 900 x *g* for 10 min. Make sure to use aerosolization-preventing caps in the centrifuge.

1.10. Pour off the supernatant into ~10% bleach solution to decontaminate. Pour 50 mL of sterile 1x PBS into the conical tube, then vortex or shake to resuspend the pellet.

1.11. Spin again at 900 x *g* for 10 min. Pour off the supernatant and resuspend the pellet in 5 mL of sterile 1x PBS. Filter through a fresh piece of miracloth into a fresh 50 mL conical tube.

1.12. Make 10-fold serial dilutions (10x, 100x, 1000x) of the fungal homogenate in 1.7 mL centrifuge tubes (e.g., for the 10x solution, mix 100 µL of the fungal homogenate with 900 µL of Tween-water).

1.13. Choose the first dilution in which the spores are not visible when it is discharged into Tween-water and use this dilution to count the number of spores using a hemocytometer.

1.14. Calculate the spore concentration in the prepared fungal homogenate (water suspension) using the following formula:

$$\text{Concentration (spores/mL)} = \text{Number of spores in middle 25 boxes} \times \text{dilution factor} \times 10^4$$

1.15. Prepare a 1 mL stock of 1.5×10^8 spores/mL in sterile 1x PBS in a 1.7 mL microcentrifuge tube. This spore preparation can be stored at 4 °C for ~4 weeks.

1.16. Prior to use in injections, mix 20 µL of the spore preparation with 10 µL of 1% sterile phenol red in a 1.7 mL centrifuge tube to achieve a final spore concentration of 1×10^8 spores/mL. Vortex thoroughly prior to injection.

NOTE: 1% phenol red solution should be filter-sterilized and stored in aliquots.

1.17. For a mock injection, mix 20 µL of 1x PBS with 10 µL of 1% sterile phenol red.

2. Preparation of agar plates for injection

2.1. Prepare 2% agarose in E3 medium and melt in a microwave.

2.2. Pour into a 100 mm x 15 mm Petri dish (~25 mL per plate), swirl to cover the plate evenly, and let cool.

2.3. Wrap the plate with paraffin film and store inverted at 4 °C.

2.4. Prior to injection, bring the plate to room temperature (RT).

2.5. Pour ~1 mL of filter sterilized 2% bovine serum albumin (BSA) onto the plate, tilt the plate to spread and cover the entire bottom, and rinse with E3.

NOTE: 2% BSA solution can be filter-sterilized and stored as 1 mL aliquots at -20 °C. 2% BSA pre-treatment prevents larvae from sticking to the surface of the agarose.

2.6. Pour E3 with buffered tricaine onto the plate and let it sit until injection.

3. Zebrafish larva hindbrain ventricle microinjection

3.1. Manually dechorionate larvae with forceps at 2 dpf in a Petri dish.

NOTE: Dechorionation can be performed anytime from 1.5 dpf until the time of injection.

3.2. Remove as much E3 as possible from the Petri dish and add buffered 300 µg/mL tricaine in E3 to anesthetize larvae.

NOTE: A stock solution of buffered 4 mg/mL tricaine in E3 can be prepared and stored at 4 ˚C. The working solution can be made by diluting 4 mL of the stock solution up to 50 mL with E3.

3.3. Use a microinjection setup supplied with the pressure injector, back pressure unit, footswitch, micropipette holder, micromanipulator, and a magnetic stand and plate, all connected to a source of compressed air (**Table of Materials**).

3.4. Open the compressed air valve and turn on the microinjector. Set the pressure to ~25 PSI, pulse duration to 6, and backpressure unit to 2 PSI.

3.5. Load a microinjection needle using a microloader pipette tip (**Table of Materials**) with about 3–5 µL of prepared PBS or spore suspension with phenol red. Mount the needle onto the micromanipulator.

NOTE: Microinjection needles can be prepared as described previously[23]. The stereo microscope used for microinjections should have an eye piece reticle to calibrate the microinjection needle. The reticle should be calibrated with a stage micrometer, and the length of the reticle scale (µm) should be determined. The diameter of the spore suspension drop that ejects from the needle is measured depending on the number of hashes (of the reticle) that overlap with the drop.

3.6. Position the micromanipulator so that the end of the needle is in view at the lowest magnification under the stereo microscope. Zoom to 4x magnification, keeping the needle in view.

3.7. Using sharp forceps, clip the end of the needle. Press the injection pedal to visualize the size of the droplet that comes out. Keep clipping back until ~3 nL of spore suspension is injected (here, this is five hashes).

3.8. Move micromanipulator and needle out of the way to avoid accidentally hitting the needle while the larvae are arranged on the injection plate.

3.9. Pour E3-Tricaine off the injection plate, then transfer ~24 anesthetized larvae to the injection plate with as little E3 possible using a transfer pipet.

3.10. Using a small tool for manipulating zebrafish larvae (i.e., hair loop tool or eyelash tool), arrange the larvae according to the direction in which they are facing. Specifically, place all facing to the right in one row, and all facing to the left in a row below.

NOTE: This arrangement is difficult if there is too much liquid on the plate, as the larvae will "float" out of place. However, too little liquid is also problematic if the injections take a long time, as the larvae can dry out or anesthesia wear off. Thus, careful attention should be paid to the amount of liquid on the plate throughout the entire microinjection process.

3.11. Adjust the microscope zoom to the lowest magnification. Bring the micromanipulator back and arrange so that the needle is close to the larvae, at a ~30˚–60˚ angle, in the middle of the field of view.

3.12. Zoom in to the highest magnification and use fine adjustment knobs to further adjust the position of the needle. Verify that ~30–70 spores are coming out of the needle by injecting the spore suspension into the liquid on the plate next to the larvae. Adjust the time and pressure on the injection setup, if necessary.

NOTE: This test should be repeated after every five to six larvae, as the number of spores coming out of the needle can increase or decrease over time.

3.13. Starting with the row in which the larvae are facing towards the needle, move the plate so that the needle is directly above and positioned near the first larvae.

3.14. Moving the needle with the micromanipulator, insert the needle through the tissue around the otic vesicle to pierce through into the hindbrain ventricle. Move the plate with the other hand as necessary to get the right orientation of the larva with the angle of the needle.

3.15. Visually verify that the end of the needle is in the center of the hindbrain ventricle, press the foot pedal to inject spores and gently retract the needle.

NOTE: The phenol red dye should primarily stay within the hindbrain ventricle. A small amount may go into the midbrain, but it should not reach the forebrain or outside the brain. If it does, the volume being injected is too large, and the pressure and time should be decreased accordingly, or a new needle should be calibrated.

3.16. Moving down the plate, inject all the larvae in that row. Then, turn the plate around and inject all larvae in the other row.

NOTE: Unsuccessfully injected or accidentally damaged larvae can be marked by 1) injecting into the yolk a couple of times to create a red mark or 2) dragging the larva out of the row with the needle.

3.17. Move needle up and out of the way again. Zoom out to a lower magnification on the microscope. The phenol red dye should still be visible in the hindbrain of each larva.

3.18. First, pull away with hair loop tool and pipette up to dispose of any larvae with unsuccessful injections. Transfer the remaining larvae into a new Petri dish by washing them off the plate with fresh sterile E3 and a transfer pipet.

3.19. Repeat as necessary for the final experimental sample number desired.

3.20. Rinse larvae at least 2x with E3 and ensure recovery from anesthesia.

3.21. To quantify survival without any further treatment, using a transfer pipette, transfer larvae into a 96 well plate (1 larva per well) in E3.

4. Establishment of injected and viable spore numbers

4.1. Immediately after injection, using a transfer pipette, randomly pick about eight of the injected larvae and transfer them to 1.7 mL centrifuge tubes (one larva per tube).

4.2. Euthanize larvae with tricaine or by placing them at 4 °C for 0.5–2.0 h.

4.3. Prepare 1 mL of 1 mg/mL ampicillin and 0.5 mg/mL kanamycin antibiotic solutions in sterile 1x PBS. The leftover solution can be stored at 4 °C and used later.

NOTE: Stock solutions of ampicillin at 100 mg/mL and kanamycin 50 mg/mL can be premade, filter-sterilized, and stored in aliquots at -20 °C. Dilute these 100x in 1x PBS to obtain the working solution.

4.4. Using a pipette, remove as much liquid as possible from the centrifuge tube, leaving the larva behind and add 90 µL of the 1x PBS with antibiotics.

NOTE: Antibiotics are used to prevent bacterial growth in GMM plates that may interfere with counting of *Aspergillus* colonies.

4.5. Homogenize larvae in a tissue lyser at 1,800 oscillations/min (30 Hz) for 6 min. Spin down at 17,000 x *g* for 30 s.

4.6. Label GMM plates (one plate per homogenized larvae). Using a Bunsen burner to create a sterile environment, pipette the homogenized suspension from one tube to the middle of the GMM plate, then spread using a disposable L-shaped spreader. Avoid spreading the homogenate against the rim.

4.7. Incubate the plates upside down at 37 °C for 2–3 days and count the number of colonies formed (CFU).

4.8. To measure the number of spores alive during the infection period, pick larvae from the 96 well plate at 1–7 days post injection (dpi) and transfer them to centrifuge tubes. Euthanize and homogenize larvae to spread on GMM plates as described in steps 4.1–4.5.

5. Drug treatment of injected larvae

5.1. After section 4, split the remaining injected larvae into two 3.5 mm dishes: one for the drug treatment and one for the control. Use about 24 infected larvae per condition.

NOTE: The 3.5 mm dishes can be treated with 2% nonfat dry milk in water, rinsed, air-dried, and stored at RT beforehand. Coating with milk will prevent larvae sticking to the plastic.

5.2. Prepare the desired drug solution and the vehicle in E3 without methylene blue in conical tubes according to the final concentration required, then mix well. For example, to monitor the survival of larvae exposed to dexamethasone, use 24 larvae (replicates) for dexamethasone and 24 for the vehicle control, such as DMSO. Prepare 5 mL of the drug solution at the required concentration. Here, 5 mL of 0.1% DMSO and 10 µM dexamethasone were used, and 24 larvae/condition were transferred to ~200 µL of the vehicle/drug solution/larvae.

5.3. Remove as much liquid as possible from one dish with a transfer pipette and add premixed

E3 containing vehicle control. Repeat with premixed E3 containing the treatment of interest for the other dish.

5.4. Using a pipette, transfer larvae into 96 well plate (one larva per well). Monitor the survival of injected larvae exposed to the vehicle or the drug for 7 days.

NOTE: Drug can be applied solely on the day of infection and kept on the larvae for the entire experiment or can be refreshed daily.

6. Daily imaging of infected larvae using the zebrafish Wounding and Entrapment Device for Growth and Imaging (zWEDGI)

6.1. Ensure that larvae are treated with 100 µM N-phenylthiourea (PTU) at 24 hpf to prevent pigmentation and that PTU is kept on the larvae for the entire experiment.

NOTE: PTU at 75–100 µM prevents pigmentation of larvae without any gross developmental defects[24]. However, PTU can interfere with some biological processes[25], and researchers should determine beforehand whether the drug may affect any processes under investigation.

6.2. Infect transgenic larvae with labeled cell populations of interest at 2 dpf with *Aspergillus* spores engineered to express a fluorescent protein, as described in section 3. Then, transfer infected larvae into wells of a 48 well plate in about 500 µL/well of E3 without methylene blue.

NOTE: A 48 well plate is used here, because it is easier to transfer larvae into and out of during repeated daily imaging.

6.3. On the day of imaging, prepare two 3.5 mm Petri dishes: one with 100 µM PTU and one with E3-tricaine.

6.4. Add E3-tricaine into the chambers of a zWEDGI device[26,27]. Under the stereo microscope, remove air bubbles from the chambers and the restraining channel using a P100 micropipette. Remove all excess E3-tricaine, so that is it only in the chambers.

6.5. Pipette up one larva from the plate using a transfer pipette. If a lot of liquid is used to remove it, pipette into a 3.5 mm dish containing E3-PTU. Then, pipette up again, using as little liquid as possible, and transfer into E3-tricaine.

6.6. Wait ~30 s for anesthetization, then transfer into the loading chamber of the wounding and entrapment device (e.g., zWEDGI).

6.7. Under the stereo microscope, position the larva. Use the P100 micropipette to remove E3-tricaine from the wounding chamber and release into the loading chamber to move the tail of the larva into the restriction channel. Ensure that larva is positioned on its lateral, dorsal, or dorso-lateral side, so that the hindbrain can be imaged with an inverted objective lens.

6.8. Image larva with a confocal microscope.

6.9. After imaging, with the P100 pipette, release E3-tricaine into the wounding chamber to push the larva from the restraining channel into the loading chamber.

6.10. Using a transfer pipette, pick up the larva and transfer it back to the Petri dish with E3-Tricaine. Using as little liquid as possible, transfer it to the Petri dish with E3-PTU. Rinse in PTU and transfer back into the 48 well plate.

REPRESENTATIVE RESULTS:

After microinjection of *Aspergillus* spores into the hindbrain of zebrafish larvae, infection outcome can be followed by multiple assays, including survival, CFUs, and live imaging. In a survival assay, the number of infected larvae surviving 1–7 dpi was monitored. When wildtype larvae were left untreated, very little death was observed, with ~80%–100% of larvae surviving the entirety of the experiment (**Figure 1**). If larvae were immunosuppressed, such as by exposure to the corticosteroid drug dexamethasone (10 µM), significantly decreased survival was observed (**Figure 1**).

When CFUs were quantified throughout the 7 day experiment from wildtype larvae infected with *A. fumigatus* spores, persistence of spores was observed, with slow clearance over time (**Figure 2A**). The number of spores surviving at 1, 2, 3, 5, and 7 dpi were normalized to the number of spores injected at 0 dpi to compare persistence and clearance across replicates (**Figure 2B**).

Transgenic fish lines expressing fluorescent proteins in leukocytes together with fluorescent protein-expressing *Aspergillus* spores can be used to visualize both leukocyte recruitment and behavior as well as fungal germination and growth[13]. When macrophages were labeled (e.g., Tg(*mpeg1:H2B-GFP*)), macrophage clustering in ~50% of larvae was typically observed, starting at 2–3 dpi (**Figure 3A**). Neutrophil (Tg(*lyz:BFP*)) recruitment was typically delayed, occurring primarily after fungal germination has occurred (**Figure 3A**). While fungal burden persisted for the whole experiment in the majority of larvae (**Figure 3A**), clearance was observed (**Figure 3B**). In some larvae, fungal burden outside of the hindbrain was also observed later in infection, due to fungal dissemination, likely in macrophages.

The area around the otic vesicle is one possible location where this dissemination could have been found (**Figure 3C**). These observations were quantified in multiple individual larvae over the course of the entire experiment (**Figure 4**). Typically, germination was seen in ~60% of larvae by 5 dpi (**Figure 4A**). Phagocyte cluster area, macrophage recruitment, and neutrophil recruitment vary both over time and across larvae, with some trending up throughout the experiment and some resolving over time (**Figure 4B,C,D**).

FIGURE AND TABLE LEGENDS:

Figure 1: Representative survival analysis of infected larvae. *Aspergillus*-infected larvae were exposed to vehicle control (DMSO) or dexamethasone (Dex), and survival was monitored. Data represent three pooled replicates. Average injection CFUs: DMSO = 30, Dex = 29 (p-value and hazard ratio were calculated by Cox proportional hazard regression analysis, ****p < 0.0001).

Figure 2: Representative CFU counts from individual infected larvae immediately after injection (0 dpi) and during infection course (2, 3, 5, and 7 dpi). Eight infected larvae were homogenized and plated to count CFU for each timepoint and replicate. (**A**) Example data from one replicate. Each point represents one larva, bars represent means for each timepoint. (**B**) CFU counts were normalized to the CFU count at 0 dpi for each replicate, and three replicates were pooled. Data were compared between experimental conditions using analysis of variance and summarized in terms of estimated marginal means and standard errors. Asterix represent statistical significance compared to CFU at 0 dpi (*p < 0.05, **p < 0.01, ****p < 0.0001).

Figure 3: Representative images from infection experiments. PTU-treated larvae with fluorescent macrophages (mpeg1:H2B-GFP) and neutrophils (lyz:BFP) were injected with RFP-expressing *A. fumigatus*. Live, infected larvae were imaged repeatedly at 2, 3, and 5 dpi on a confocal microscope. Maximum intensity Z projection images are displayed. Schematics of larvae indicate location of imaging for each panel. All scale bars represent 100 μm, except for the inset scale bars, which are 25 μm. (**A**) Images shown are from a single larva taken at 2, 3, and 5 dpi, representing a typical infection progression. Insets show fungal germination at days 3 and 6. (**B**) Representative image of subset of larvae that can clear the infection, with low fungal burden and not much inflammation at 5 dpi. (**C**) Representative image of subset of larvae in which infection disseminates out of the hindbrain at later timepoints. In this image, fungus, macrophages, and neutrophils can be found around and below the otic vesicle.

Figure 4: Representative quantification of imaging experiments. Images from experimental setup in **Figure 3** were analyzed for fungal germination and leukocyte recruitment. (**A**) Larvae were scored for the presence of germinated spores on each day, and the percentage of larvae with germination was calculated. (**B,C,D**) Each individual larva is represented as a different color line. Phagocyte cluster appearance and size (B), macrophage recruitment (C), and neutrophil recruitment (D) were followed over the course of the 5 day experiment for each larva.

DISCUSSION:

The infection model described here is beneficial for analyzing the host immune responses, host-pathogen interactions, and fungal pathogenesis[12-15]. This information can be derived from the high-resolution imaging of fluorescent-labeled pathogens and host cells[13], larval survival, and CFU persistence over time.

The microinjection technique is critical to the success of this protocol and may need to be adjusted when using different microinjection equipment and setups. In particular, the pressure and time of injection are two major variables and can be adjusted to ensure that the volume

ejected by the needle is ~3 nL. The size of the needle as determined by clipping it with forceps also regulates the number of spores being injected; although, a larger opening can cause tissue damage to the larva. On the other hand, too small of an opening will not allow the relatively large spores (>2 μm) out and can lead to needle clogging. If this occurs, the needle can be reclipped to have a slightly larger opening.

Other protocols for microinjection of bacteria utilize PVP-40 to help maintain a homogenous injection mixture, but we have not found any advantage in using this carrier with *Aspergillus* spores. Clogging of the needle can be mitigated by vortexing the fungal preparation thoroughly to break any clumps prior to loading the needle. Sometimes, a clog in the needle can also be dislodged by temporarily increasing the pressure or injection time and triggering the microinjector while the needle is in the liquid surrounding the larvae. The pressure and injection time should then be decreased again to previous levels. In other cases, a clog cannot be removed, and a new needle needs to be loaded and recalibrated.

This protocol is designed to inject ~30–70 spores per larva. It is known that based on the concentration of the spore preparation and the volume injected, this number is quite low. However, it has been empirically found that this is the number of spores injected under these conditions. Why this difference occurs is unknown, but it may be due to spore clumping in the needle. Our own attempts to inject larger numbers of spores have largely been unsuccessful.

To ensure about 30–70 spores are being injected and maintain the consistency of the injections throughout all the larvae, check the number of spores by injecting onto the E3 surrounding the larvae. Repeat this every five to six larvae throughout all the injections. If the spore count seems to change, the pressure and/or injection time can be adjusted to inject a consistent number of spores across multiple larvae. However, care should be taken that the injection dose remains primarily in the hindbrain and does not fill the midbrain and forebrain.

To ensure a localized infection, the spore suspension should be contained within the hindbrain ventricle. This can be visualized by the phenol red staining just after the injection, though the red color diffuses with time. For injections, the region around the otic vesicle is used to pierce through and reach the ventricle at a 45°–65° angle. This area has no main blood vessels, causes less tissue damage, and heals instantly. If the skin over the ventricle is pierced, the spore suspension can be leaked out, because the needle that must be used for *Aspergillus* spore injections is larger than that used for bacterial suspensions. Unsuccessfully injected or accidentally damaged larvae can be marked by injecting into the yolk a couple of times to create a red mark or by dragging the larva out of the row with the needle. After a set of injections is complete, these larvae should be removed and disposed of before the rest are washed off the plate. E3 without methylene blue is used to anesthetize larvae prior to injection and also keep larva after the injections, because methylene blue is anti-fungal.

At the time of injection, CFU counts represent the number of viable spores within the infected host. However, if the spores germinate into hyphae, these can be broken up into separate viable “fungal units” during homogenization and can give rise to multiple colonies. Or, an

unbroken multicellular hypha can give rise to a single colony, resulting in an averaged, but imprecise, representation of the fungal burden. This can be mitigated by combining the CFU counts with longitudinal microscopy of individual larvae, which provides visual data of the fate of injected spores.

Compared to the mammalian system, the zebrafish larva infection model is particularly significant due to its optical accessibility. The recruitment and response of innate immune cells can be visualized within a live intact host. This can be incorporated with genetic or chemical inhibition of molecular targets to analyze how each target affects the macrophage or neutrophil reaction against *Aspergillus* spores in a live animal.

While the zebrafish larva *Aspergillus* infection model continues to be instrumental in describing different aspects of IA[12-20,22], there are other areas of expansion. From the host side, it is used to describe cellular level immune responses, but this can be expanded to analyze immune mechanisms at the molecular level by combining it with targeted morpholino, CRISPR, stable mutant lines, or chemical exposure. One caveat is that homologues for all known mammalian innate immune pathway components have not been identified in zebrafish.

From the pathogen side, virulence of different species and strains have been described. A promising avenue of future research is the use of mutant *Aspergillus* strains to test how specific genes or proteins contribute as virulence factors. Thereby, novel anti-fungal drugs can be developed to target these proteins. Current anti-fungal drugs have low efficacy in human patients and there is growing resistance to these drugs in fungi[28]. This in vivo model can be used to investigate why these drugs fail and as an intermediate model to test the efficacy of novel anti-fungal drugs. Overall, the findings discovered using this model can facilitate future development of effective treatments for *Aspergillus*-infected patients.

ACKNOWLEDGMENTS:

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DISCLOSURES:

No conflicts or financial interests to disclose.

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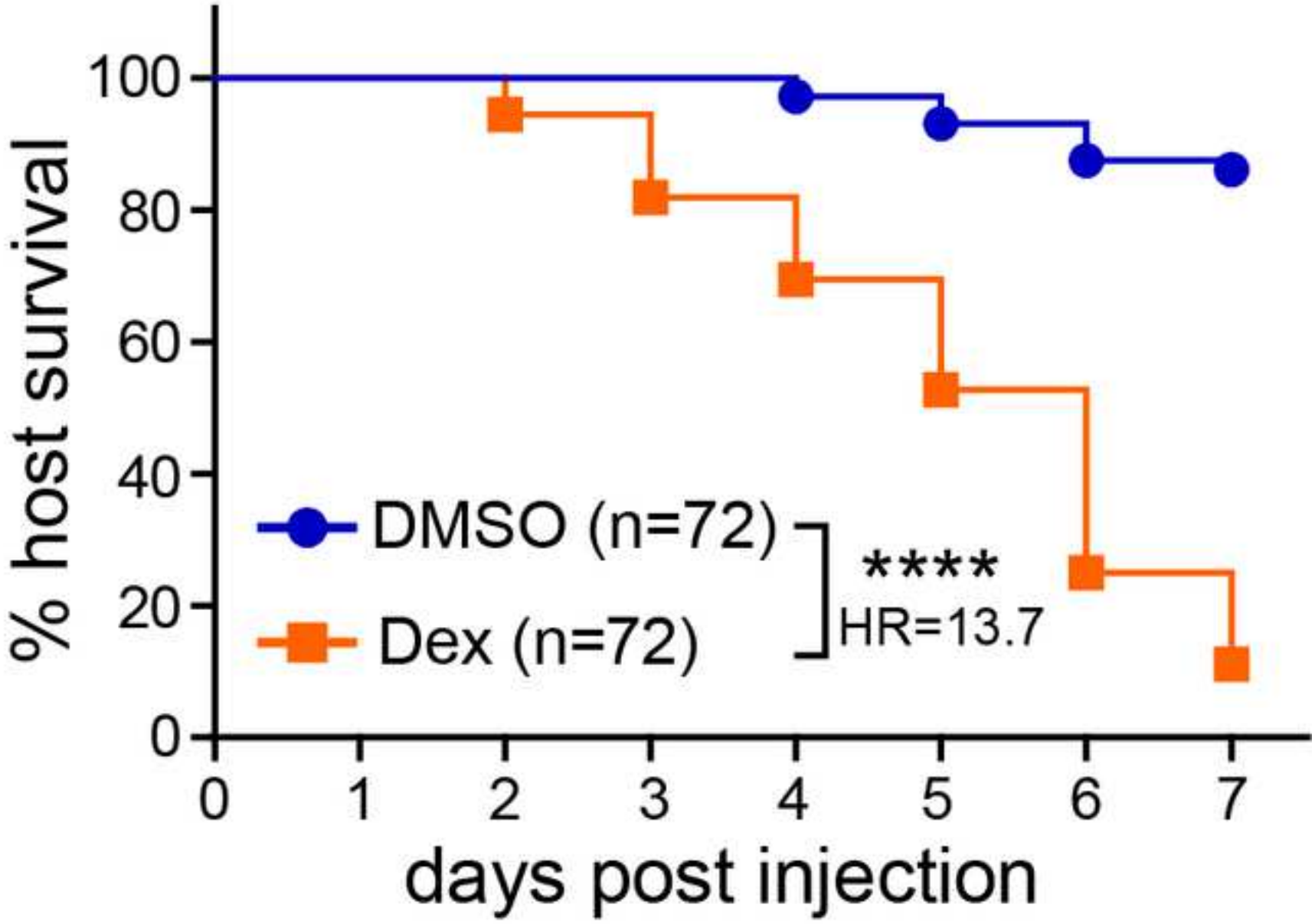
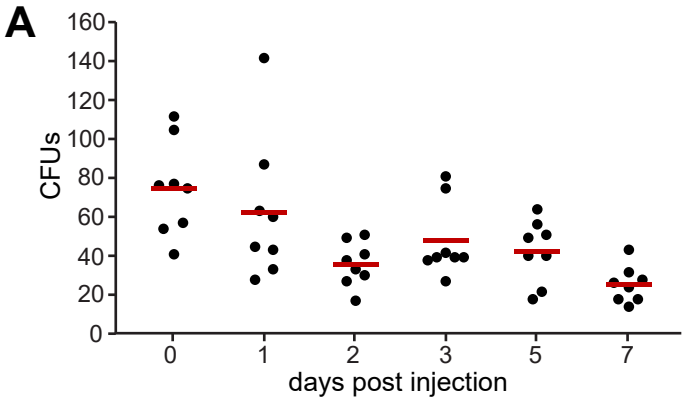
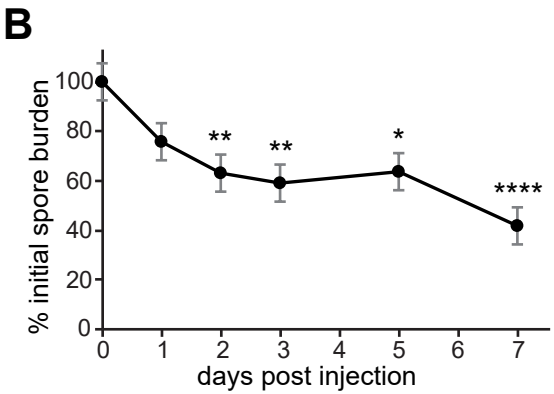
Figure 1



Figure

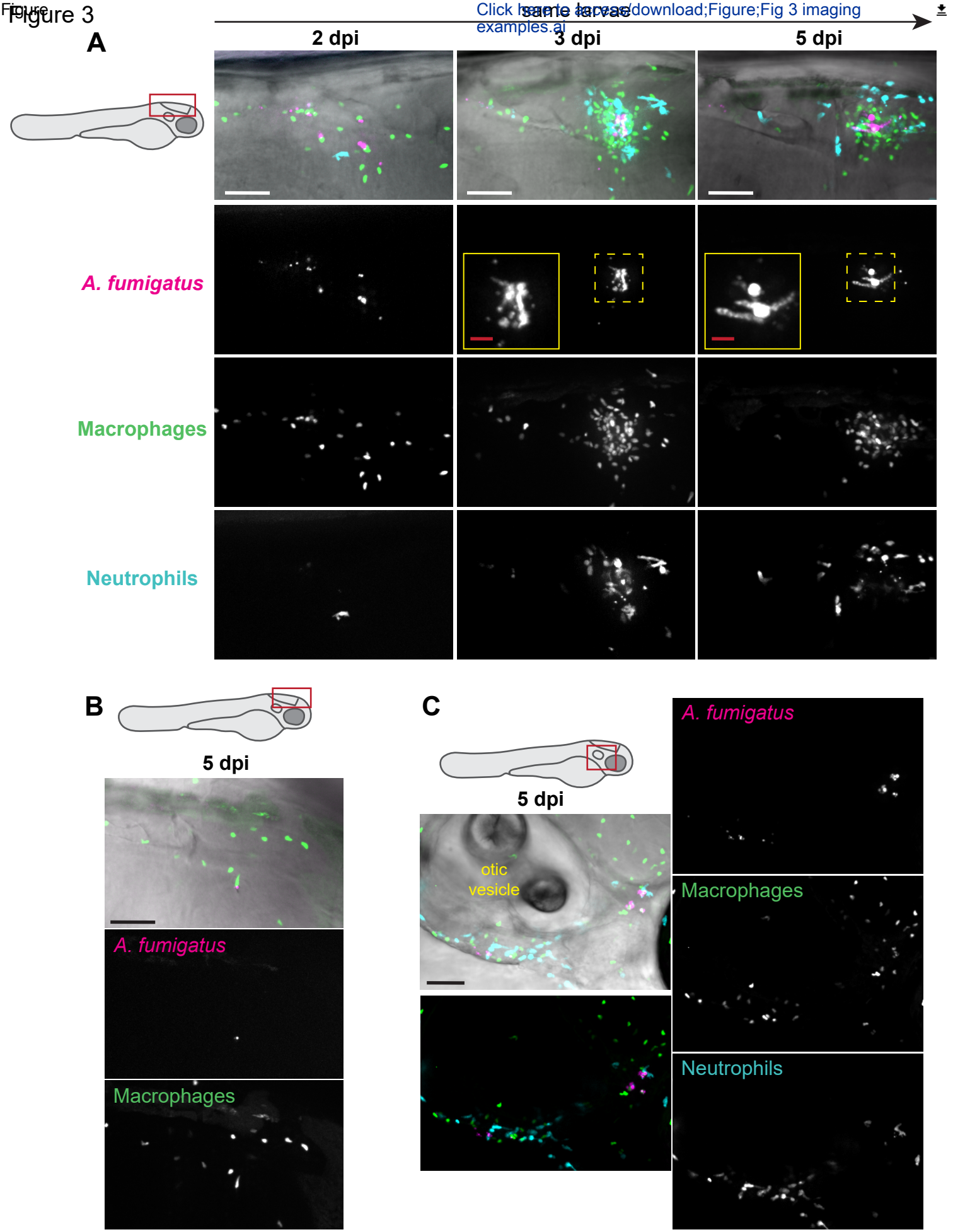
Figure 2





Figure

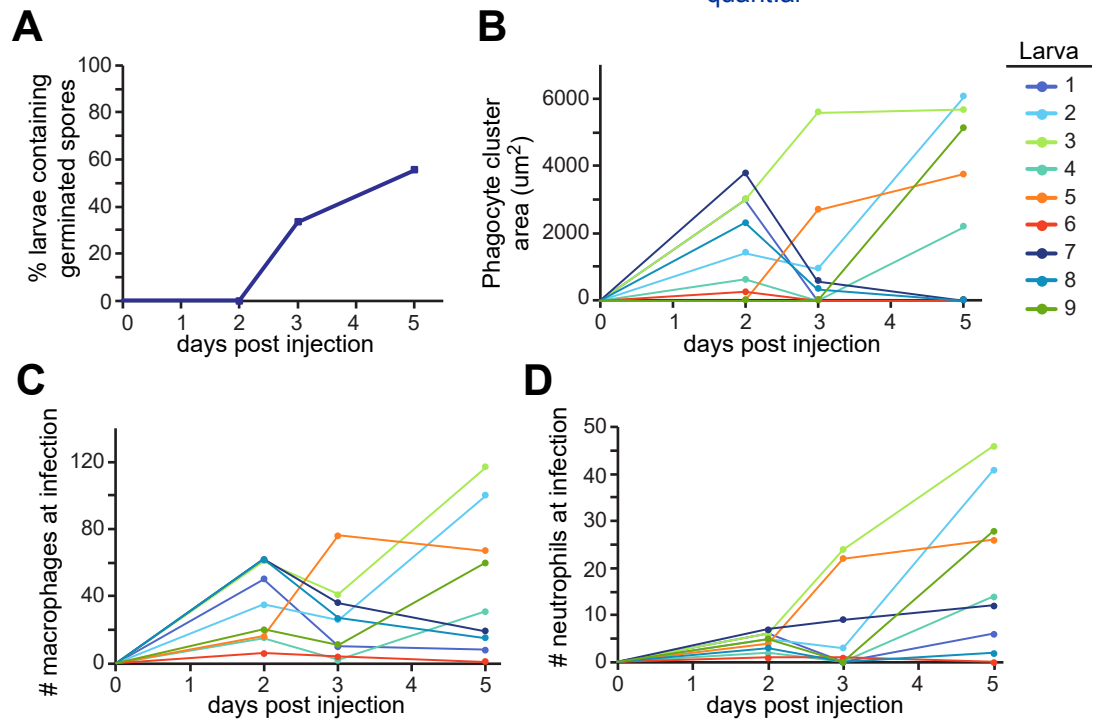
Figure 3



Figure

Figure 4

Click here to access/download;Figure;Fig 4 image quant.ai



Equipment

Dumont forceps #5	
Eyepiece reticle	
Microinjector setup:	Back pressure unit
	Footswitch
	Micro pipet holder kit
	Pressure injector
Micromanipulator setup:	Micromanipulator
	Magnetic stand and plate
Needle puller	
Stereomicroscope	
Tissuelyser II	

Material

Agarose
Ampicillin sodium salt
BSA, fraction V
Kanamycin sulfate
L spreaders
Microcapillary needles (no filament)
Microloader pipet tips
Miracloth
N-phenylthiourea
Phenol red, 1% solution
Tricaine (Ethyl 3-aminobenzoate, methanesulfonic acid salt)
Tween-20

Media and Solutions

E3 media:	60x E3
	1x E3
Tricaine stock solution	
Glucose minimal media (GMM) agar:	GMM agar
	20x Nitrate salts
	Trace elements (TE)

Company	Catalog Number
Roboz Surgical Instrument Co.	RS-5045
Microscope World	RETR10
Applied Scientific Instrumentation	BPU
Applied Scientific Instrumentation	FTSW
Applied Scientific Instrumentation	M-Pip
Applied Scientific Instrumentation	MPPI-3
Narashige (Tritech)	M-152
Tritech	MINJ-HBMB
Sutter Instrument	P-97
Nikon	SMZ-745
Qiagen	85300

Company	Catalog Number
Fisher	BP160-500
Fisher	AAJ6380706
VWR	AAJ65855-22
Fisher	AAJ1792406
Fisher	14 665 230
World Precision Instruments (WPI)	TW100-3
VWR	89009-310
VWR	EM475855-1R
Fisher	AAL0669009
Fisher	57254
Fisher	AC118000500
Fisher	BP337-500

Components/Recipe

17.2 g NaCl, 0.76 g KCl, 2.9 g $CaCl_2$, 4.9 g $MgSO_4 \cdot 7H_2C$

16.7 ml 60x stock, 430 ul 0.05 M NaOH, to 1 L with H_2O (

2 g Tricaine, 5 g $Na_2HPO_4 \cdot 7H_2O$, 4.2 ml 60X E3, to 500 m

10 g Glucose (Dextrose), 50 ml 20x Nitrate salts, 1 ml TE,

120 g $NaNO_3$, 10.4 g KCl, 10.4 g, $MgSO_4 \cdot 7H_2O$, 30.4 g,

2.20 g $ZnSO_4 \cdot 7H2O$, 1.10 g H_3BO_3, 0.50 g $MnCl_2 \cdot 4H_2C$

Comments/Description

For calibrating needles, used in Stereomicroscope

To homogenize larvae

Comments/Description

To load the needle with Aspergillus suspension
To filter Aspergillus suspension
To prevent pigmentation

To anesthetize larvae

), to 1 L with H_2O

optional: + 3 ml 0.01% methylene blue)

ml with H_2O, pH to 7.0-7.5 with NaOH

to 1 L with H2O, pH to 6.5 with NaOH, + 16 g Agar, autoclave

KH_2PO_4, to 1 L with H_2O, autoclave

), 0.16 g $FeSO_4 \cdot 7H_2O$, 0.16 g $CoCl_2 \cdot 6H_2O$, 0.16 g CuSO4 · $5H_2O$, 0.11 g $(NH_4)_6Mo_7O_{24} \cdot$

· $4H_2O$, 5.00 g Na_2EDTA, to 100 ml with H_2O, dissolve stirring overnight, autoclave

We would like to thank the Editor and the Reviewers for their insightful comments, which have helped us to improve the manuscript significantly. We hope that our manuscript is now acceptable for publication in JoVE. Please find our replies to each specific comment below in bold.

Editorial comments:
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.

Thank you for bringing this to our attention. We have reviewed and corrected all the mistakes.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points throughout.

Thank you for emphasizing this and for the detailed explanation. We have made the changes accordingly.

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Thank you for highlighting this. Suggested points of each step are now mentioned as Notes.

4. Please use complete sentences throughout.

Corrected.

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

Thank you for pointing this out. The protocol is now revised.

6. Please ensure that individual steps of the protocol should only contain 2-3 actions per step. We cannot have paragraph of text in the protocol section.

Done.

7. Please ensure you answer the “how” question, i.e., how is the step performed?

Thank you for pointing this out. We have made changes in the protocol to explain ‘how’ to perform each step.

8. At what time point after injection, do you perform step 4 and 5?

Step 4 is performed immediately after injection followed by step 5. This is now mentioned more clearly in the protocol. Please see lines 297 and 332.

9. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted the most critical points of the protocol that are required to successfully replicate the technique.

10. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

All the images used in this manuscript are original and not obtained from previous publications.

11. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have edited the Discussion section, moving and condensing some less critical suggestions to Notes in the protocol and expanding discussion of the critical steps of the protocol, troubleshooting, and limitations.

12. Please do not abbreviate the journal titles in the references section.

Thank you for pointing this out. Complete journal titles are now mentioned for all the references.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:

Thrikawala et al., present methods for establishing and quantifying Aspergillus fumigatus infection in zebrafish. For establishing infection, they describe approaches for microinjection of the hindbrain. For quantification of fungal burden, they present methods for whole-animal CFU enumeration as well as tracking of fungal growth and clearance over time using longitudinal confocal microscopy of individual infected fish.

Overall the manuscript is well-written and logically presented. The subject is important: opportunistic fungal infections result present an increasing clinical challenge. Modeling of fungal infection in zebrafish allows opportunities to study host-pathogen interactions directly in a vertebrate model with unprecedented detail. Use of such models will be central to better understanding the underlying biology of infection and developing new treatments.

Infection by microinjection is technically challenging, the video format for this methods paper is highly appropriate for conveying key steps in the process. Previous video articles demonstrating bacterial infection (e.g. Benard, 2012 JOVE) have been incredibly useful to the field and thus highly cited. As such, my suggested improvements are minor.

Major Concerns:
None

Minor Concerns:

1) Although vehicle control are suggested for drug treatments, no mock infection control is included. Because fungal infection requires the use of larger needles than bacterial infection, there is often more damage caused to the embryo during microinjection, which can lead to secondary infection from environmental microbes and death of the embryo. Similarly, tissue damage from microinjection can result in inflammatory responses that need to be considered when quantifying recruitment of host cells.

Thank you for bringing this up. The *Aspergillus* spores are suspended in 1X PBS, therefore, we use PBS as the mock infection diluted 2:1 in 1% sterile phenol red. We have included that in the protocol. Please see line 170.

2) L72: The authors cite their own work assessing drug activity using this model. This model has also been used to measure the activity of newly developed compounds designed to enhance host responses (Jones et al., 2019, Frontiers in Immunology). This paper should also be cited.

Thank you for bringing this to our attention. It is now cited in the Introduction (line 72) and the Discussion (line 521).

3) L154 typo: the stock solution of Tricaine is stated as "4 ml/mL"

Thank you for pointing this out. It is now corrected.

4) The appropriateness of CFU versus imaging approaches could be discussed further. Because A. fumigatus lay down single septum between cells, physical dissociation of hyphae and plating is likely to underestimate the number of viable fungal cells. Similarly, inadequate dissociation of hyphae could result in a single colony arising from a hypha containing multiple cells. As such, direct microscopy provides the best estimation of fungal burden for hyphal pathogens, and the optical accessibility of the zebrafish provides a key advantage in this regard.

This is an excellent suggestion. We have added a paragraph in the Discussion elaborating the limitations of CFU and the advantage of direct microscopy regarding fungal burden. Please see lines 508-514.

Reviewer #2:

Manuscript Summary:
In their manuscript, the authors describe a microinjection technique for Aspergillus spores into the hindbrain of zebrafish larvae. This technique allows detailed study of host-pathogen interactions important in invasive aspergillosis (IA). The rationale behind the methodology is sound and the disease model has already proven its usefulness for the study of IA. The protocol is described in great detail and should be sufficient to allow researchers to successfully perform this technique. I have no major concerns and only minor questions/suggestions.

Major Concerns:
N/A

Minor Concerns:

- Line 91: It might be a good addition to point out that researchers should obtain relevant approval from their local animal care and ethical committees. Local differences in regulations can occur (e.g. European animal experimentation laws prevent the use of zebrafish larvae after 5 days post fertilization without explicit authorisation).

Thank you for pointing this out. We have clarified this point, now in lines 91-94.

- Notes are being used throughout the manuscript to provide additional information. Following the description of PTU treatment (lines 267 - 269) it would be important to mention that blocking pigmentation with PTU also induces side effects that could interfere with the biological process under investigation. If pointed out, researchers can then make their own assessment whether this is an issue for their experimental setup.

Thank you for pointing this out. We have now included this as a Note (lines 364-366).

- Lines 133 - 137: While injecting, spores are in sterile 1X PBS (0.66X PBS after addition of phenol red). Other microinjection methods suggest the use of 2% PVP in PBS as a carrier solution to prevent precipitation of bacteria (e.g. Benard et al, Jove, 2012). Is precipitation not an issue when injecting Aspergillus spores?

We do think that *Aspergillus* spores can clump in the needle, but, in our hands, we have not seen any difference in clumping/clogging with or without PVP. We have now added this information to the Discussion section. Please see lines 477-479.

- Line 136, line 176 & line 189: Injecting ~3 nl of 1 x 10^8 spores/ml does not equate to 30-70, but to ~300 spores. What explains this difference?

Great question. Mathematically, it should be around 300 spores. However, empirically, what we actually get out of the needle is ~30-70. We are not really sure why, possibly due to spore clumping higher up in the needle. This discrepancy is now discussed in the Discussion section (lines 485-489).

- For a typical drug treatment experiment, how many animals per treatment group should we aim for?

We usually use 24 larvae for survival experiments. We have now included this in section 5.1, line 333.