Video Article

Non-invasive Imaging of the Innate Immune Response in a Zebrafish Larval Model of *Streptococcus iniae* Infection

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Abstract

The aquatic pathogen, *Streptococcus iniae*, is responsible for over 100 million dollars in annual losses for the aquaculture industry and is capable of causing systemic disease in both fish and humans. A better understanding of *S. iniae* disease pathogenesis requires an appropriate model system. The genetic tractability and the optical transparency of the early developmental stages of zebrafish allow for the generation and non-invasive imaging of transgenic lines with fluorescently tagged immune cells. The adaptive immune system is not fully functional until several weeks post fertilization, but zebrafish larvae have a conserved vertebrate innate immune system with both neutrophils and macrophages. Thus, the generation of a larval infection model allows the study of the specific contribution of innate immunity in controlling *S. iniae* infection.

The site of microinjection will determine whether an infection is systemic or initially localized. Here, we present our protocols for otic vesicle injection of zebrafish aged 2-3 days post fertilization as well as our techniques for fluorescent confocal imaging of infection. A localized infection site allows observation of initial microbe invasion, recruitment of host cells and dissemination of infection. Our findings using the zebrafish larval model of *S. iniae* infection indicate that zebrafish can be used to examine the differing contributions of host neutrophils and macrophages in localized bacterial infections. In addition, we describe how photolabeling of immune cells can be used to track individual host cell fate during the course of infection.

Video Link

The video component of this article can be found at http://www.jove.com/video/52788/

Introduction

Streptococcus iniae is a major aquatic pathogen that is capable of causing systemic disease in both fish and humans[1]. While *S. iniae* is responsible for large losses in the aquaculture industry, it is also a potential zoonotic pathogen, capable of causing disease in immunocompromised human hosts with clinical pathologies similar to those caused by other streptococcal human pathogens. Given its similarities with human pathogens, it is important to study *S. iniae* disease pathogenesis in the context of a natural host. An adult zebrafish model of *S. iniae* infection revealed robust infiltration of host leukocytes to the localized site of infection as well as a rapid time to host death, a time too short to involve the adaptive immune system[7]. In order to gain an in-depth look into the innate immune response to *S. iniae* infection *in vivo*, it is necessary to use a model that is more amenable to non-invasive live imaging.

The larval zebrafish has a number of advantages that make it an increasingly attractive vertebrate model for studying host-pathogen interactions. Zebrafish are relatively inexpensive and easy to use and maintain compared to mammalian models. Adaptive immunity is not functionally mature until 4-6 weeks post fertilization, but larvae have a highly conserved vertebrate innate immune system with complement, Toll-like receptors, cytokines, and neutrophils and macrophages with antimicrobial capabilities including phagocytosis and respiratory burst[2-6,8-11]. In addition, the genetic tractability and optical transparency of the embryonic and larval stages of development allow for the generation of stable transgenic lines with fluorescently labeled immune cells making it possible to examine host-pathogen interactions in real time *in vivo*. The generation of these transgenic lines using a photoconvertible protein such as Dendra2 allows for the tracking of individual host cell origin and fate over the course of infection[12].

When developing a zebrafish larval infection model, the chosen site of microinjection will determine whether an infection is initially localized or systemic. Systemic blood infections into the caudal vein or Duct of Cuvier are most commonly used to study microbial pathogens in zebrafish and are useful for studying interactions between host and microbial cells, cytokine responses, and differences in virulence between pathogen strains. For slower growing microorganisms, early injection into the yolk sac of an embryo at the 16-1,000 cell stage can be used to generate a systemic infection[13,14], with the optimal developmental stage for microinjection of a slow-growing microorganism found to be between the 16

to 128 cell stage[15]. However, yolk sac injections of many microbes at later stages of host development tend to be lethal to the host due to the nutrient-rich environment for the microbe and lack of infiltrating leukocytes[16-18].

A localized infection usually results in directed migration of leukocytes towards the site of infection that can be easily quantified with non-invasive imaging. This type of infection can allow for dissection of the mechanisms that mediate leukocyte migration as well as investigation of different migratory and phagocytic capabilities of various leukocyte populations. Localized infections are also useful when examining differences in virulence between bacterial strains as well as studying microbe invasion mechanisms since physical host barriers must be crossed for a localized infection to become systemic. Zebrafish are typically raised at temperatures of 25-31 °C[19], but they can also be maintained at temperatures as high as 34-35 °C for studies of the invasiveness of certain human pathogens with strict temperature requirements for virulence[20,21].

Many different sites have been used to generate an initially localized bacterial infection including the hindbrain ventricle[22], dorsal tail muscle[18], pericardial cavity[23], and otic vesicle (ear)[5,16,24]. However, it has been found that injection of bacteria into tail muscle can cause tissue damage and inflammation independent of the bacteria, which may skew results when investigating leukocyte response[13]. Although less damage is associated with injection into the hindbrain and although it is initially devoid of leukocytes in young embryos, the hindbrain ventricle steadily gains more immune cells over time as microglia take up residence. The hindbrain ventricle is also a more difficult location to image. The otic vesicle is a closed hollow cavity with no direct access to the vasculature[25,26]. It is normally devoid of leukocytes, but leukocytes can be recruited to the otic vesicle in response to inflammatory stimuli such as infection. It is also a preferred site of microinjection of bacteria in zebrafish aged 2-3 days post fertilization (dpf) because of the ease of imaging and the visualization of the injection. Therefore, we chose the otic vesicle as our site of localized bacterial infection.

Protocol

Adult and embryonic zebrafish were maintained in accordance with the University of Wisconsin-Madison Research Animal Resources Center.

1. Preparing Microinjection Needles

1. Prepare thin wall glass capillary injection needles (1.0 OD/0.75 ID) using a micropipette puller device with the following settings: air pressure 200, heat 502, pull 90, velocity 80, time 70, air time at start of pull 5, air time at end of pull 5.
2. Using fine tweezers, break off the tip of the pulled needle so that the tip opening has a diameter of approximately 10 µm.

2. Preparing Larval Injection Dishes

1. Rinse a gel comb with lane width of approximately 4-5 mm in sterile water and allow to dry.
2. Prepare a 1.5-2% high melt agarose solution in E3 medium[19] and microwave until the solution is clear. Once cooled, pour some of the agarose into a Petri dish (100 x 15 mm) and swirl, adding just enough agarose to completely cover the bottom of the dish.
3. Once the agarose layer has solidified, place the rinsed and dried gel comb on top so that the non-combed end is just resting on the top of the Petri dish and the combed end is touching the agarose. Ensure that the comb is as horizontal as possible, creating a 30° angle with respect to the bottom agarose layer.
4. Pour an additional small amount of agarose at the interface between the comb and bottom agarose layer so that the fresh agarose layer covers the wells of the comb. Allow to cool completely before removing the comb. Using a pipet tip, remove any overhanging pieces of agarose from the wells.
5. Pour E3 medium on top of the injection mold and store at 4 °C.
6. Before each use, replace with fresh medium and warm injection ramps at 28.5 °C for at least an hour before injection.
7. Immediately prior to injections, replace the E3 on the injection ramp with E3 medium containing 200 µg/ml ethyl 3-aminobenzoate (tricaine).

3. Preparing *S. iniae* Inoculum

1. Prepare and autoclave Todd Hewitt broth medium supplemented with 0.2% yeast extract and 2% proteose peptone (THY+P): 30 g/L Todd Hewitt, 2 g/L yeast extract, 20 g/L proteose peptone. For agar plates, add 14 g/L agar.
2. Prepare bacterial cultures the night before infections by pipetting a 100 µl aliquot of frozen bacterial stock into 10 ml of THY+P broth in a sealed 15 ml tube. Incubate overnight without agitation at 37 °C. After 14-16 hr of growth, use the overnight culture either to make freezer stocks or prepare for use in injections.
3. Make freezer stocks by placing 1 ml of the overnight culture in 500 µl of 80% glycerol in a 1.7 ml centrifuge tube. To avoid freeze-thaw cycles, make 100 µl one-use aliquots from this mix and store at -80 °C.
4. For *S. iniae* used in infections, dilute the overnight culture 1:100 for a total volume of 10 ml culture by adding 0.1 ml of overnight culture to 9.9 ml of THY+P broth. Grow at 37 °C without agitation for approximately 4-5 hr. Monitor the optical density (OD) at 600 nm using a Nanodrop spectrophotometer and harvest the bacteria in mid-logarithmic phase when the OD600 nm reaches 0.250-0.500. An OD600 nm of 0.250 corresponds to approximately 10^8 colony forming units (CFU)/ml.
5. Pellet 1 ml of the bacterial culture in a 1.7 ml centrifuge tube at 1,500 x g for 5 min. Resuspend in 1 ml of fresh PBS and repeat. Measure the OD600 nm of the bacteria in PBS, pellet, and resuspend in PBS to achieve the desired concentration.
6. To aid in the visualization of microinjection, add phenol red to the bacterial suspensions prior to injection for a final concentration of 0.1%.
7. For experiments involving the injection of heat-killed bacteria, heat the bacteria in PBS at 95 °C for 30 min. Confirm that the heat-killing process reduced the number of viable bacteria to undetectable levels by plating an injection volume (approximately 1 nl) on solid THY+P agar plates and incubating overnight at 37 °C.

4. Labeling *S. iniae* with a CellTracker Red Fluorescent Dye

1. To label living bacterial cells, prepare a stock solution of a CellTracker fluorescent dye or equivalent. As the dye used comes in 20 x 50 µg aliquots of powder and needs to be resuspended in DMSO, add 7.3 µl DMSO to the tube to obtain a 10 mM stock concentration.
2. Test a range of dye concentrations (*e.g.,* 0.5-25 µM) on the bacteria to determine the lowest optimal concentration that stains the cells. Rapidly dividing cells and longer experiments may require a higher concentration of dye.
    1. Pellet 1.0 ml of bacterial culture in a 1.7 ml tube by centrifugation as described above (section 3). Resuspend the pellet in 1 ml fresh PBS and add the appropriate volume of dye to the bacterial culture.
    2. Incubate without agitation at 37 °C for 30 min. Spin down the bacteria and resuspend in 1 ml of pre-warmed THY+P broth and incubate without agitation for an additional 30 min at 37 °C.
    3. Spin down the bacteria and wash two times in PBS before measuring the OD600 nm and diluting the bacteria for microinjection as detailed above (section 3). We typically inject approximately 100 CFU in 1 nl injection volume for our studies of leukocyte recruitment and phagocytosis.

5. Preparation of Zebrafish Larvae for Infections

1. Set up breeding pairs the night before and collect embryos as described by Rosen *et al.*[27] Incubate embryos in E3 medium at 28.5 °C until ready to infect.
2. For zebrafish that will be imaged, prevent the development of pigment (melanization) by adding N-Phenylthiourea (PTU) to the E3 medium at 24 hr post fertilization (hpf) for a final concentration of 0.2 nM.
3. For infections involving embryos aged 2 dpf, dechorionate embryos manually with a pair of fine tweezers. Alternatively, dechorionate embryos by removing E3 and replacing with pronase (2 mg/ml) for about 5 min or until gentle pipetting breaks embryos out of the chorion. Most larvae should have hatched naturally by 3 dpf.
4. Anesthetize zebrafish several minutes prior to infection by placing dechorionated larvae into E3 medium containing 200 µg/ml tricaine.

6. Otic Vesicle Injection of *S. iniae* into Three Day Old Larvae

1. Turn on the microinjector and set the time range to “millisecond”. Open the valve on the carbon dioxide tank to let gas into the line. The pressure of the microinjector should read approximately 20 PSI. Adjust the pressure in the microinjector unit by clockwise turning of the black knurled knob for increased pressure or counterclockwise turning for decreased pressure.
2. Vortex the prepared *S. iniae* culture in phenol red and PBS and use a microloader tip to load 2-3 µl of the culture into a pulled capillary injection needle.
3. Mount the loaded needle on a micromanipulator connected to a magnetic stand and position it under a stereomicroscope so that the needle is at approximately a 45-65° angle with respect to the base of the microscope.
4. Press on the foot pedal of the microinjector to dispense a drop of the inoculum onto the tip of the needle. Measure the diameter of the drop using the scale bar in the ocular lens of the microscope.
    1. Alternatively, estimate the diameter of the drop by injecting a volume into a drop of mineral oil on a glass microscope slide with a scale bar. The diameter of the drop should be approximately 0.10 mm, which is about a 1 nl volume.
    2. Adjust the drop size by adjusting the duration setting on the microinjector or by clipping off more of the needle tip with fine tweezers. Note that the injection time should be between 20 - 35 msec to avoid causing too much tissue damage.
5. Using a plastic transfer pipet, transfer 12 anesthetized larvae into each well of the injection mold.
6. Use a glass rod, hair loop, or plastic tip to gently position the larvae so that the heads are pointed towards the back of the microscope and the yolk sacs are against the left side of the well. Point the left ear of the larva up towards the ceiling.
7. Looking through the ocular lens of the stereomicroscope, use the knobs on the micromanipulator to line up the loaded needle with the otic vesicle so that both are in the same field of view. Pierce the outer epithelial layer of the otic vesicle with the needle tip so that the needle tip is just inside the vesicle.
8. Press on the foot pedal to inject 1 nl of the desired dose of *S. iniae*. Be sure to use a low enough pressure so as not to rupture the cavity. If the injection is successful, the otic vesicle, but not the surrounding tissue, should fill with the phenol red inoculum (**Figure 1 Ai**). Immediately remove any mis-injected fish from the injection plate.
9. Carefully retract the needle out of the larva and move the injection plate by hand so that the next larva is in view. Do this under 5X magnification.
   Note: Retraction of the needle may result in the deposition of some bacteria outside of the otic vesicle, which may skew survival and leukocyte recruitment results. To avoid this, it is recommended to inject fluorescent dye-labeled bacteria and visually scan injected larvae under a fluorescent microscope to remove any larvae where this has occurred. A correctly injected larva is shown in (**Figure 1 Bi**).
10. To ensure the injection volume/inoculum remains the same over the course of the experiment, inject a drop of the bacterial suspension into a 1.7 ml centrifuge tube containing 100 µl of sterile PBS after every 48th embryo. Plate the 100 µl on THY+P agar plates at 37 °C overnight to determine the CFU in the injection volume.
11. When the entire group of 12 larvae on the injection ramp has been injected, carefully use a plastic transfer pipette to remove the larvae from the wells and place them into a new Petri dish (35 x 10 mm^2). Remove the tricaine solution and replace with approximately 2 ml of fresh E3 medium to allow the larvae to recover.
12. Pipet injected larvae into single wells of a 96-well plate and incubate at 28.5 °C. Monitor larvae over time for survival in these plates or remove later for imaging or CFU counts.

7. Sudan Black Staining of Neutrophils

Note: The following steps can be done at room temperature in a small Petri dish (35 x 10 mm^2) on an orbital shaker unless otherwise stated. For each step, the amount of reagent used is approximately 2 ml per dish, using just enough liquid to completely cover the larvae.

1. For fixation of infected larvae, use a glass Pasteur pipette to remove E3 medium and replace with an ice-cold 4% paraformaldehyde in PBS solution. Immediately place the fish at 4 °C to euthanize. Fix overnight at 4 °C.
2. Wash 3 times in PBS for 5 min each.
3. Stain with Sudan Black (0.18% stock diluted 1:5 in 70% ethanol, 0.1% phenol) for 30 min to 5 hr. Use a stereomicroscope to check if neutrophil granules have taken up the stain.
4. Perform a series of 5 min washes starting with a single wash in 70% ethanol followed by a single wash of a 1:1 ratio of 70% ethanol and PBS, followed by a final wash in PBS.
5. Rehydrate into PBS plus 0.1% tween (PBT).
6. To clear pigment from the larvae, wash in 1% potassium hydroxide/1% hydrogen peroxide for about 6-10 min. Monitor the sample closely and after about 5 min, check the larvae under a stereomicroscope to see how much of the pigment has cleared. If the solution is left on too long, the yolk sacs will swell and burst.
7. Wash 3 times for 5 min each in PBS.
8. Store the samples in either PBS or PBT at 4 °C for up to 1 week until ready to image and count.
9. To image Sudan Black-stained larvae, transfer the fish into PBT and then into 80% glycerol and place onto a single cavity depression slide. Position fixed larvae under a stereomicroscope using a pipette tip. Image using a color digital camera on a stereomicroscope (**Figure 1 Aii-iv**).

8. Enumeration of Viable Bacteria from Infected Larvae

1. Euthanize larvae at the desired times post infection in an ice-cold 200-300 mg/L solution of tricaine in E3 medium.
2. Pipet euthanized larvae into 1.7 ml centrifuge tubes. Remove the tricaine solution and replace with 100 µl of 0.2% Triton X-100 in PBS (PBSTx).
3. Homogenize larvae by passing up and down 10 times through a 27 G needle.
4. Prepare serial dilutions of homogenates in sterile PBS. For example, if the infectious dose is 100 CFU, pipet 100 µl of the homogenate into 900 µl sterile PBS. Using a new pipet tip, transfer 100 µl of the diluted homogenate into 900 µl sterile PBS.
5. Plate 100 µl of the dilutions on Columbia CNA agar for selective isolation of gram-positive bacteria, and incubate at 37 °C for 48 hr. This medium will provide greater selection than the THY+P agar plates, which will support growth of both gram-negative and gram-positive bacteria.
6. On plates with fewer than 500 individual colonies, count the number of colonies and multiply by the dilution factor to determine the number of CFU.

9. Fixation of Larvae for Imaging

1. Fix larvae in an ice-cold 4% paraformaldehyde in PBS solution as described in section 7.
2. At room temperature, wash 3 times for 5 min each in PBS before imaging.

10. Preparation of Larvae for Live Imaging

1. Prepare a 1.5% low-melting-point agarose solution in E3 by heating in the microwave until the solution is clear.
2. Place agarose solution in a 55 °C water bath to let it cool but not harden.
3. Add tricaine to the agarose to a final concentration of 0.016%.
4. Using a plastic transfer pipette, place 4-5 anesthetized larvae in a glass bottom dish on the stage of a stereomicroscope.
5. Remove the tricaine and agarose solution from the 55 °C water bath and let it cool at room temperature for 1-2 min. While the agarose is cooling, remove as much liquid from the anesthetized larvae as possible.
6. Pour the cooled agarose into the dish until about half of the surface is covered. Swirl the dish to spread the agarose. Note that if the agarose is too hot, it will kill the larvae. Also, if too much agarose is added to the dish, the objective lens of the microscope may hit the bottom of the dish when focusing through the agarose.
7. Using a transfer pipette, pick up the larvae that have floated to the sides of the dish and pipet them back into the center.
8. Under the stereomicroscope, gently position the larvae as desired with a hair loop, a long pipette tip or a glass rod. For imaging of the otic vesicle, position the larvae so that the left otic vesicle is flat against the bottom of the dish.
9. Let the agarose cool for about 10 min before moving the dish. The larvae may shift positions if the agarose is not solid. Gently pipet some tricaine and E3 solution to the top of the agarose layer to keep it moist.

11. Confocal Imaging of Infection

1. Place the glass bottom dish with larvae onto the stage of an inverted microscope with an FV-1000 laser scanning confocal system.
2. Set the pinhole to 200-300 µm and using a numeric aperture 0.75/20X objective lens, set z-stacks with 3-6 µm slices.
3. Use continuous line scanning to adjust the laser power and detector gain for each channel.
4. Using sequential line scanning for each fluorescence channel (*e.g.,* 488 and 543 nm) and differential interference contrast (DIC), conduct a time lapse movie of the left otic vesicle every 3 min for 2-6 hr to observe initial recruitment and phagocytosis by neutrophils and macrophages. For longer time courses, place a lid on the Petri dish to prevent evaporation and drying out of the agarose.

5. Acquire still images of fixed (**Figure 1B**) or live (**Figure 2**) larvae at 20X or 40X magnification.

12. Photoconversion of Dendra2-labeled Leukocytes at the Otic Vesicle

Note: Dendra2 can be photoconverted from green to red fluorescence by focusing a 405 nm laser (50-70% laser power should be sufficient) on the region of interest (ROI) for 1 min. Below is the step-by-step protocol used for the FV-1000 laser scanning confocal system:

1. Visualize the sample using a z-stack scan with the 488 nm and 543 nm lasers. Use continuous line scanning to adjust the laser power and detector gain. Check to make sure there is no accidentally photoconverted red fluorescence.
2. On the “Image Acquisition Control” window, under “Stimulus Setting” select the “Use Scanner” tool and choose “main”. Select the 405 nm laser and set it at 70% power. Using the circle option, define the ROI in the otic vesicle.
3. Under “Stimulus Start Setting” select “Activation in series” with a preactivation of 1 frame and an activation time of 60,000 msec. On the “Acquisition Setting” window under the “Time Scan heading”, choose 2 intervals of 00:01:00 (one for pre- and one for post- photoconversion). Note: After the time lapse series scan is complete, the Dendra2 should have been photoconverted to its red fluorescent state.
4. Scan the sample by a z-stack using the 488 nm and 543 nm lasers to visualize the photoconverted red fluorescence as well as any remaining green fluorescence (**Figure 3**).

Representative Results

Microinjection of *S. iniae* into the otic vesicle (**Figure 1** and **Figure 2**) results in an initially localized host response. When injected correctly, the bacteria should only be seen in the otic vesicle and not in the surrounding tissue or blood. This can be visualized during microinjection using phenol red dye (**Figure 1A**). Alternatively, if labeled bacteria are injected, a quick scan of infected larvae immediately post injection can confirm the bacteria are only in the otic vesicle and not the surrounding tissue (**Figure 1B**). Although a dose as little as 10 CFU wild type *S. iniae* is able to establish a lethal infection within 24-48 hr post infection (hpi), injection of 1,000 CFU of an avirulent strain, *cpsA*, does not result in lethal infection and that strain seems unable to proliferate in the host[24]. Thus, this localized infection model is able to differentiate between bacterial strains of altered virulence.

Microinjection sites of initially localized infection are useful for studying leukocyte chemotaxis. Leukocyte recruitment can by quantified by either Sudan Black staining of neutrophil granules (**Figure 1A**) or formaldehyde fixation of fluorescent transgenic lines (**Figure 1B**). To visualize the recruitment and phagocytic capabilities of immune cells, we used transgenic lines expressing the green fluorescent protein Dendra2 specifically in macrophages *Tg(mpeg1:dendra2)*[24] or neutrophils *Tg(mpx:dendra2)*[12]. When *S. iniae* is injected into the otic vesicle of 3 dpf larvae, both neutrophils and macrophages are rapidly recruited within the first 2 hpi (**Figure 1**). However, when performed correctly, microinjection of PBS into the otic vesicle does not result in the same robust recruitment of host leukocytes (**Figure 1**). In addition to recruitment, live confocal time lapse imaging of fluorescent transgenic lines injected with fluorescently-labeled bacteria reveals the phagocytic capabilities of both neutrophils and macrophages. Red dye-labeled *S. iniae* can be found inside both neutrophils and macrophages (**Figure 2**). Using the photoconvertible protein Dendra2 to label neutrophils or macrophages allows for non-invasive photolabeling for tracking individual cell fate over the course of the infection. Macrophages that were recruited to the otic vesicle at 5 hpi were photoconverted and then tracked over the following 24 hr. Although some photoconverted cells remain in the otic vesicle or head region, some can also be found disseminated throughout the body of the larvae (**Figure 3**).

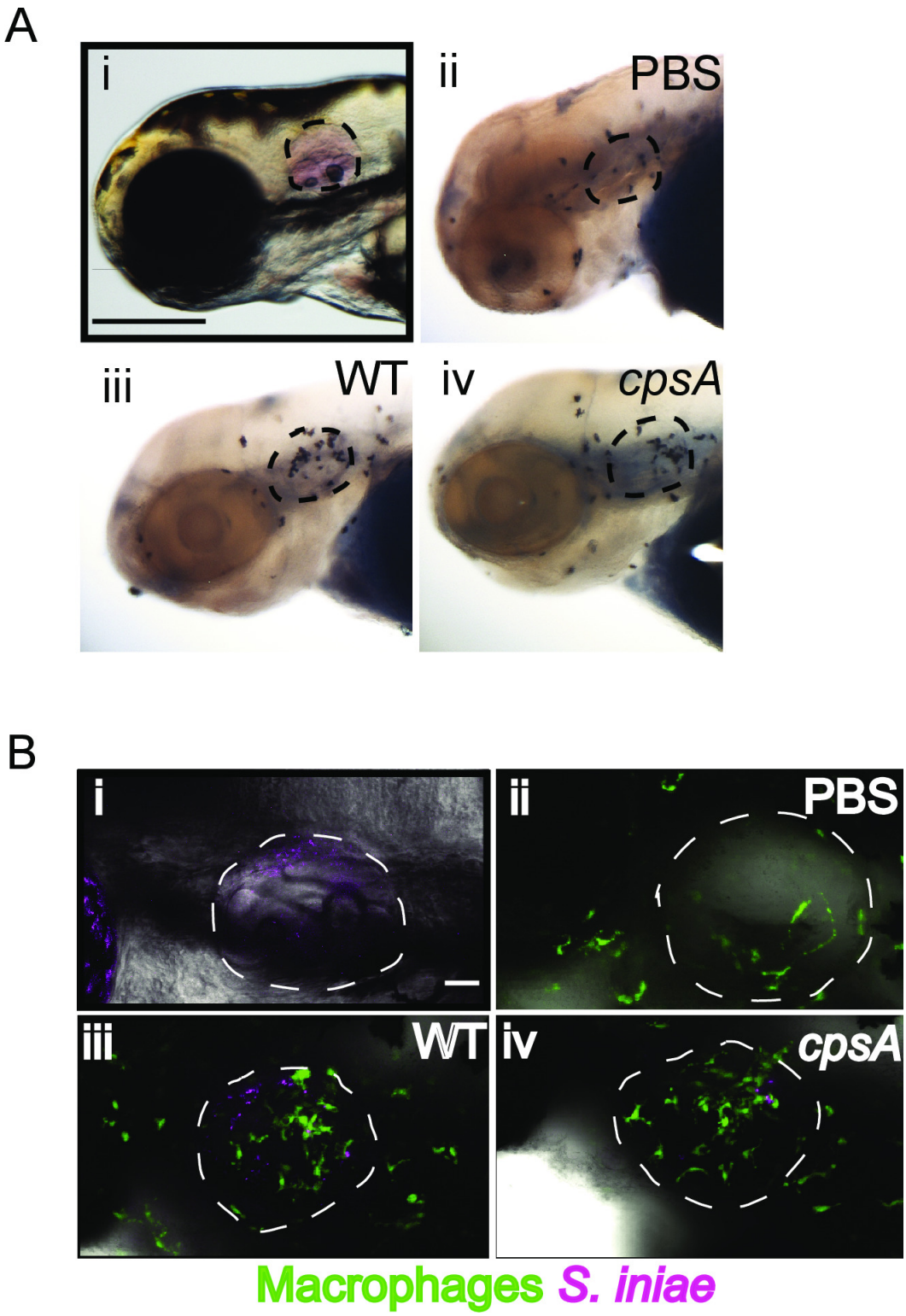

Figure 1: Leukocyte recruitment to otic vesicle infection with *S. iniae*. (**A**) Neutrophil recruitment to *S. iniae* infection. (**i**) Successful injection of a phenol red-labeled inoculum into the otic vesicle. (**ii-iv**) Sudan Black staining of larvae for investigation of neutrophil recruitment at 2 hpi. PBS mock-infected larvae show little recruitment of neutrophils to the otic vesicle (**ii**) whereas infection with either wild type *S. iniae* or the *cpsA* mutant results in robust neutrophil recruitment (**iii**, **iv**). Scale bar, 300 µm. (**B**) Macrophage recruitment to *S. iniae* infection. (**i**) Successful microinjection of red-labeled *S. iniae* (depicted in magenta) into the otic vesicle. (**ii-iv**) Fluorescent confocal images of microinjected transgenic *mpeg1:dendra2* larvae fixed at 2 hpi. PBS mock-infected larvae show little macrophage recruitment (**ii**), but larvae infected with CellTracker Red-labeled (depicted in magenta) wild type *S. iniae* or the *cpsA* mutant show robust macrophage recruitment to the otic vesicle at 2 hpi **(iii, iv)**. Scale bar, 30 µm. Please click here to view a larger version of this figure.

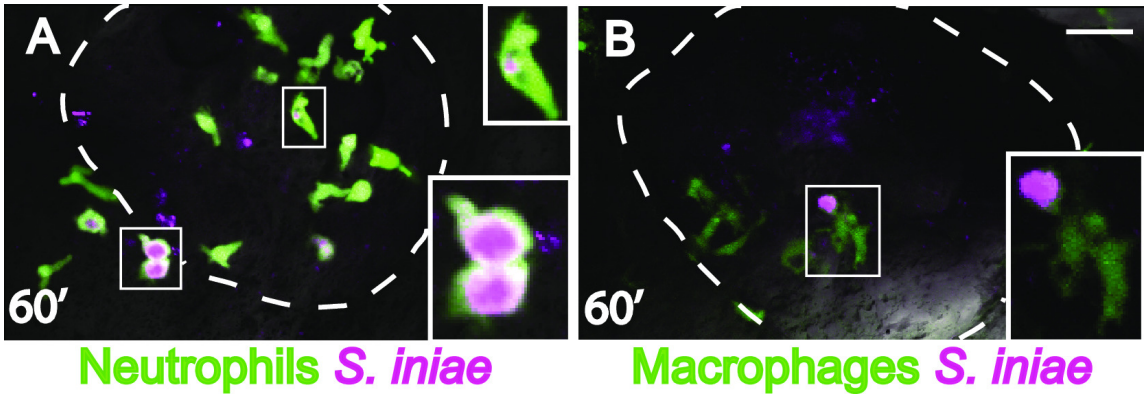

Figure 2: Phagocytosis of *S. iniae* by phagocytes in the otic vesicle. Transgenic *mpx:dendra2* (**A**) or *mpeg1:dendra2* (**B**) larva infected with red-labeled *S. iniae* (depicted in magenta) and imaged at 60 min post infection using a laser scanning confocal microscope. Scale bar, 30 µm. Please click here to view a larger version of this figure.

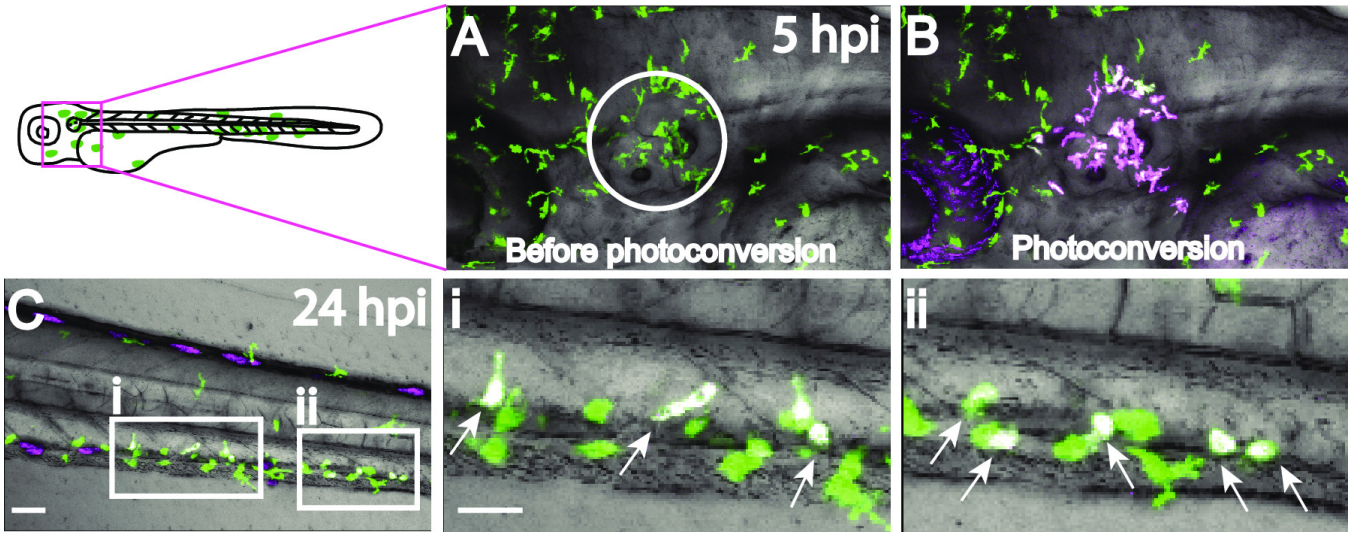

Figure 3: Photoconversion of macrophages at the otic vesicle 5 hpi with *S. iniae*. Macrophages (depicted in green) at the otic vesicle, designated by the circle (**A**), were photoconverted (**B**) using a 405 nm laser on a confocal microscope and tracked over time. By 24 hpi, photoconverted macrophages (depicted in magenta) have migrated as far as the trunk/caudal hematopoietic tissue (**C**); scale bar, 50 µm. Higher magnifications of the boxed regions in C are shown in (**i**) and (**ii**), scale bar 30 µm; arrows point to photoconverted macrophages. Photoconverted cells appear white because of the merged 543 nm red fluorescence and any remaining 488 nm green fluorescence. Please click here to view a larger version of this figure.

Discussion

The infection method used here is useful for the study of the host immune response to an initially localized infection in 2-3 dpf embryos and larvae. The focus of an inflammatory stimulus, such as infection, in a closed cavity such as the otic vesicle allows for the study of neutrophil and macrophage chemotaxis and phagocytosis. One caveat of injecting bacteria into the otic vesicle is that the ability of neutrophils to efficiently phagocytose bacteria in fluid-filled cavities may be dependent on the particular microbe. Although *Escherichia coli* and *Bacillus subtilis* are not easily phagocytosed by neutrophils in the otic vesicle[28], we have found that neutrophils are able to phagocytose both *Pseudomonas aeruginosa* and *S. iniae* in this location[16,24]. Localized infection is also useful when studying the invasiveness of various pathogens. In order to cause a systemic infection following injection into a closed cavity such as the otic vesicle, the microbe must be able to traverse physical host barriers. Alternatively, different pathogens may rely on host cells for transportation and dissemination from the initial site of infection. This makes localized injections useful for comparing strains of altered virulence.

During microinjection, to avoid bacteria settling and clogging the needle, change needles either after each condition or after about 50 larvae. This will help ensure the suspension in the needle is more uniform. If settling of bacteria in the needle seems to be a problem, a mix of PBS, 2% PVP40, and 10% glycerol may help keep a homogenous suspension. To ensure that each larva is being injected with approximately the same number of bacteria, check CFU counts by homogenizing a larva immediately following injection and plating the homogenate on CNA agar. CNA agar will select for the growth of culturable gram-positive bacteria, not only *S. iniae*, but it will provide an idea of how consistent the injection doses are between each individual larva. With a target inoculum of 100 CFU per larva, there are typically between 75-150 colonies on a CNA plate. The number of non-*S. iniae* gram-positive colonies growing on a CNA plate is probably very low, since most PBS injected fish usually result in between 0-20 colonies. At later time points during the infection, it is not uncommon for there to be variation of up to half a log in colony

counts between larvae infected with the same infectious dose. This could represent slight differences between individual larva in their ability to control the infection or could reflect differences in the amount of culturable gram-positive bacteria in the larvae or E3 medium.

While injecting into the otic vesicle, it is important not to inject too large a volume or with too high a pressure as this may cause the cavity to rupture. Injection volumes should be kept to approximately 1 nl. If the needle is too large, microinjection can cause damage to the surrounding tissue, which may affect the recruitment of host leukocytes to the site of infection or may allow bacteria to leak out of the otic vesicle. It is also important to confirm injection into the correct space. If the needle is inserted too deep, it may poke through the otic vesicle or it may hit blood vessels flowing around the otic vesicle. This may lead to the deposition of bacteria into the blood stream or outside the vesicle, which may alter host responses.

It is also possible that when the needle is extracted, some bacteria may be accidentally deposited outside the otic vesicle as shown by Colucci-Guyon *et al.*[28], but we find this to only be the case in less than 5% of the injections. Trying to inject into the center of the otic vesicle may help avoid this situation. In a successful infection, the otic vesicle should fill up with the phenol red dye, but the dye should not leak out into the surrounding tissues. Any mis-injected larvae should be immediately discarded. Injecting labeled bacteria is a useful way to confirm a successful injection (**Figure 1B**). One of the disadvantages of using a CellTracker dye is that this dye will become diluted as the bacteria divide, eventually preventing the bacteria from being visualized under fluorescence. We chose a red dye because we used green-labeled neutrophil and macrophage transgenic lines for the majority of our studies.

Dendra2 is a photoconvertible protein derived from octocoral *Dendronephthya sp*. which can be photoconverted from a green to a red fluorescent state with a 405 nm laser[29]. This photoconversion is a noninvasive way to mark cells and track their fate over the course of infection. Leukocytes recruited to the site of infection can be photoconverted and followed over time to monitor their dissemination throughout host tissues (**Figure 3**). Alternatively, leukocytes could be photoconverted prior to infection and then monitored post-microinjection to determine the origin of cells recruited to the site of infection. Labeling bacteria and immune cells allows for the study of leukocyte-pathogen interactions *in vivo* including recruitment and phagocytosis (**Figure 1B** and **Figure 2**). Distinguishing the red-labeled *S. iniae* from the red fluorescence of a photoconverted leukocyte is difficult, so a different fluorescent CellTracker dye could be used. This would be particularly useful to determine which of the photoconverted immune cells that leave the otic vesicle contain *S. iniae*.

Future applications of the otic vesicle infection method could include measuring the speed and directionality by which neutrophils and macrophages move in response to various infections. The infection kinetics can also be studied to see which cell types are the first to arrive to a site of infection and which cell types are most robustly recruited in addition to where the cells originate or where they disseminate[30]. In addition to studying the recruitment to an initially localized infection, this infection model can be used to study the function of host immune system components during initial infection. Antisense morpholino oligonucleotides that target specific RNAs can be used to knock down expression of host immune components including Toll-like receptors, cytokine receptors and leukocytes, and CRISPR-Cas or TALEN technology can be used to create genetic mutants. Gene expression using RNAseq or qPCR can also be used to characterize the expression of certain host genes in response to infection.

Disclosures

The authors declare that they have no competing financial interests.

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