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Video Article

Infection of Zebrafish Embryos with Intracellular Bacterial Pathogens

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Abstract

Zebrafish (*Danio rerio*) embryos are increasingly used as a model for studying the function of the vertebrate innate immune system in host-pathogen interactions [1]. The major cell types of the innate immune system, macrophages and neutrophils, develop during the first days of embryogenesis prior to the maturation of lymphocytes that are required for adaptive immune responses. The ease of obtaining large numbers of embryos, their accessibility due to external development, the optical transparency of embryonic and larval stages, a wide range of genetic tools, extensive mutant resources and collections of transgenic reporter lines, all add to the versatility of the zebrafish model. *Salmonella enterica* serovar Typhimurium *(S. typhimurium)* and *Mycobacterium marinum* can reside intracellularly in macrophages and are frequently used to study host-pathogen interactions in zebrafish embryos. The infection processes of these two bacterial pathogens are interesting to compare because *S. typhimurium* infection is acute and lethal within one day, whereas *M. marinum* infection is chronic and can be imaged up to the larval stage [2, 3]. The site of micro-injection of bacteria into the embryo (**Figure 1**) determines whether the infection will rapidly become systemic or will initially remain localized. A rapid systemic infection can be established by micro-injecting bacteria directly into the blood circulation via the caudal vein at the posterior blood island or via the Duct of Cuvier, a wide circulation channel on the yolk sac connecting the heart to the trunk vasculature. At 1 dpf, when embryos at this stage have phagocytically active macrophages but neutrophils have not yet matured, injecting into the blood island is preferred. For injections at 2-3 dpf, when embryos also have developed functional (myeloperoxidase-producing) neutrophils, the Duct of Cuvier is preferred as the injection site. To study directed migration of myeloid cells towards local infections, bacteria can be injected into the tail muscle, otic vesicle, or hindbrain ventricle [4-6]. In addition, the notochord, a structure that appears to be normally inaccessible to myeloid cells, is highly susceptible to local infection [7]. A useful alternative for high-throughput applications is the injection of bacteria into the yolk of embryos within the first hours after fertilization [8]. Combining fluorescent bacteria and transgenic zebrafish lines with fluorescent macrophages or neutrophils creates ideal circumstances for multi-color imaging of host-pathogen interactions. This video article will describe detailed protocols for intravenous and local infection of zebrafish embryos with *S. typhimurium* or *M. marinum* bacteria and for subsequent fluorescence imaging of the interaction with cells of the innate immune system.

Video Link

The video component of this article can be found at http://www.jove.com/video/3781/

Protocol

1. Prepare Injection Needles

1. Prepare borosilicate glass microcapillary injection needles (Harvard Apparatus, 300038, 1 mm O.D. × 0.78 mm I.D.) using a micropipette puller device (Sutter Instruments Inc., Flaming/Brown p-97 with the following settings for a longer tip: air pressure 400; heat 610; pull 40; velocity 50; time 30 and with the following settings for a shorter tip: air pressure 500; heat 510; pull 100; velocity 200; time 60).
2. Break off the needle tip with fine tweezers to obtain a tip opening diameter of 5-10 µm. It is advisable to bevel the needle tip opening at a 45 degrees angle with a microgrinder (Narishige Inc., EG-400) to yield a sharper tip. This will facilitate puncturing the epidermal layer and thus result in more reproducible injections with less tissue damage than with blunt needles. Longer tipped needles are preferred for caudal vein (step 5) and Duct of Cuvier (step 6.1) injections and shorter tipped needles are preferred for the other injection protocols (steps 6.2-6.6).

2. Prepare *S. typhimurium* Inoculum

1. Plate out *S. typhimurium* from a -80 °C glycerol stock onto LB agar plates (with appropriate antibiotics to select for fluorescence expression vectors) and incubate overnight at 37 °C.

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2. Pick individual fluorescently positive colonies and resuspend them to the desired concentration (see protocols 5 and 6) in sterile phosphate-buffered saline (PBS), optionally containing 0.085% (v/v) phenol red (Sigma-Aldrich) to aid visualization of the injection process. Directly use the fresh suspension for the injection or prepare glycerol stocks. To prepare glycerol stocks, spin down the freshly made injection stock with the desired concentration of bacteria and concentrate the stock by resuspending the pellet in half the starting volume in sterile 20% (v/v) glycerol (Sigma-Aldrich) in PBS. Store the glycerol stock at -80 °C. Dilute the glycerol stock 1:1 (v/v) prior to injection in sterile PBS, optionally containing 0.17% phenol red.
3. Vortex the bacterial suspension well to avoid clumping.
4. Load the inoculum into the microcapillary needle using a microloader tip (Eppendorf, 5242956.003).
5. Due to the relatively large size of *S. typhimurium* bacteria and their bright Ds-RED fluorescence when using the pGMDs3 expression vector [3] (strain available upon request), individual bacterial cells can easily be counted with a fluorescence stereomicroscope in order to set the injection dose. To this end, inject 1 nL into a drop of PBS on an agar plate, count the fluorescent bacteria, and calculate the injection volume that is required to obtain the desired bacterial dose (preferably keep the injection volume between 1-2 nL). Inject the embryos with *S. typhimurium* via the selected route (see protocols 5 and 6).

3. Prepare *M. marinum* Inoculum

1. Keep the *M. marinum* strain growing on Difco Middlebrook 7H10 agar (BD and company) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC, BD and company), 0.5% glycerol, and with appropriate antibiotics to select for fluorescence expression vectors (strains available upon request [9]), so there is always a fresh stock.
2. Pick a colony of *M. marinum* and resuspend it in Difco Middlebrook 7H9 broth (BD and company) supplemented with 10% albumin-dextrose-catalase (ADC, BD and company) and 0.05% Tween 80 (Sigma-Aldrich) and the appropriate antibiotics. Check that the optical density (OD) at 600 nm is 0.2 - 0.3 and let it grow statically overnight at 28.5 °C. The generation time of *M. marinum* is approximately 4-6 h, varying according to the strain.
3. Measure the OD at 600 nm again on the day of injections. An OD600 of 1 corresponds to approximately 10^8 *M. marinum*/mL (this may vary according to the bacterial strain used and thus should be based on a growth curve for the particular strain).
4. Harvest the bacteria when they are in logarithmic phase (do not let the OD600 exceed 1.00) by centrifuging and washing them three times in sterile PBS.
5. Measure the OD600 again of the bacterial suspension in PBS, spin down, and resuspend the bacteria to the desired concentration (see protocols 5 and 6) in PBS or in 2% Polyvinylpyrrolidone (PVP40) in PBS (w/v), which improves homogeneity of the bacterial suspension. Phenol red (Sigma-Aldrich) may be added to a concentration of 0.085% to aid visualization of the injection process. Directly use the fresh suspension for the injection or prepare glycerol stocks. To prepare glycerol stocks spin down the freshly made injection stock with the desired concentration of bacteria and concentrate the stock by resuspending the pellet in half the starting volume in sterile 20% glycerol in PBS. Store the glycerol stock at -80 °C. Dilute the glycerol stock 1:1 (v/v) in sterile PBS, optionally containing 4% PVP40 and/or 0.17% phenol red.

4. Prepare Zebrafish Embryos for Injections

1. Set up zebrafish breeding pairs and collect embryos as shown in another video article[10]. Keep embryos in a Petri dish filled with egg water (60 µg/mL Sea salts; ca. 60 embryos/dish) and incubate at 28.5 °C.
2. If required, add 0.003% *N*-Phenylthiourea (PTU, Sigma-Aldrich) to the egg water when the embryos are approximately 12 hpf to prevent melanization.
3. Around 24 hours post fertilization (hpf), dechorionate the embryos with fine tweezers (Fine Science Tools Inc., Dumont #5 Forceps - Inox Biology.
4. Keep the embryos in a Petri dish filled with egg water and with a layer of 1% agarose on the bottom to prevent embryos from sticking to the plastic surface.
5. Anesthetize the embryos with 200 µg/mL buffered 3-aminobenzoic acid (Tricaine, Sigma-Aldrich) approximately 10 min prior to injections.

5. Intravenous Injection of Bacteria into One-day old Embryos

1. Stage the embryos at 28 hpf [11] by checking for consistent blood circulation, beginning of pigmentation in the eye, a straight tail, and the heart being positioned just ventrally to the eye.
2. Anesthetize the embryos, see step 4.5.
3. Load the needle with the bacterial inoculum using a microloader tip.
4. Mount the loaded needle onto a micromanipulator (Sutter Instrument, MM-33) connected to a stand (World precision Instruments, M10L magnetic stand) and position it under a stereo microscope (Leica M50, achromat 1x objective 0,15 NA, transmitted light base TL ST). Set the injection time to 0.2 s and the compensation pressure to 15 hPa (Eppendorf, Femtojet). Adjust the injection pressure between 700 and 900 hPa to obtain the correct injection volume for the needle used. Adjust the drop size to match the desired diameter with the help of a scale bar on a microscope slide or in the ocular. For size determination the drop can be injected into mineral oil on a microscope slide, or the size can be estimated by injecting into the air, which leaves the drop hanging on the needle tip. The radius of a drop of 1 nL is 0.062 mm ($V = 4/3 \pi r^3$).
5. Set the micromanipulator with the loaded needle into the correct position prior to injecting (i.e. approximately at a 45° angle with respect to the injection plate surface) and only move it back and forth to inject.
6. The anesthetized embryos are pipetted onto a flat 1% agarose injecting plate and excess egg water is removed, allowing surface tension to hold the embryos in place during injections. Use a hair loop tool (**Figure 2**) to line up the embryos. Orient the injection plate by hand during injections to place the embryos into the preferred position for inserting the needle, i.e. with their tails pointing towards the needle tip.
7. Place the needle tip directly above the caudal vein close to the urogenital opening (**Figure 1A**), pierce the periderm with the needle tip and inject the desired dose of bacteria; we use ca. 250 cfu of *S. typhimurium* wild type (wt) strain SL1027, containing the Ds-RED expression vector pGMDs3, and ca. 120 cfu of *M. marinum* strain Mma20. The injected bacterial suspension will follow the blood flow through the caudal

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vein towards the heart. Monitor if the injection was performed correctly by checking for an expanding volume of the vascular system directly after the pulse [2]. For dose-response experiments, 2-3 consecutive injections can be performed without extracting the needle.
8. Frequently check that the injection volume remains the same during the experiment. To provide a control for the consistency of the injections throughout the experiment, inject a drop of bacteria directly into a sterile PBS drop on bacterial growth medium after approximately every 30th embryo injection. Plate out this drop and count the bacterial colonies after incubation to determine the colony forming units (cfu) in the injection volume.
9. Use a fluorescence stereomicroscope (protocol 7) to observe individual fluorescent *S. typhimurium* cells circulating in the bloodstream directly after injection, and discard embryos that are not properly injected. Individual fluorescent *M. marinum* bacteria cannot be observed by stereo fluorescence microscopy directly after injections, but fluorescent aggregates of infected cells should be visible by 2 days post infection (dpi) and grow larger over time.

6. Alternative Routes of Infection

1. Duct of Cuvier injection: line up anesthetized embryos (2-3 dpf) on a flat 1% agarose injecting plate as for caudal vein injections (see 5.6). Orient the injection plate by hand during injections to place the embryos into the preferred position for inserting the needle, i.e. diagonally under a 45° angle so that the Duct of Cuvier can be approached by the needle tip from the dorsal side of the embryo (**Figure 1B**). Insert the needle into the starting point of the Duct of Cuvier just dorsal to the location where the duct starts broadening over the yolk sac and inject 100-200 bacteria (1-3 nL). The injection is correct if the volume within the duct expands directly after the pulse and the yolk sac is not ruptured. As for caudal vein injections (see 5.7), several consecutive injections can be performed without extracting the needle.
2. Hindbrain ventricle injection: line up anesthetized embryos (32 hpf) on a flat 1% agarose injecting plate such that the embryos are positioned with their dorsal side towards the needle tip. Insert the needle into the hindbrain ventricle from an anterior position without touching the neuroepithelium (**Figure 1C**) and inject 20-100 bacteria (0.5-1 nL). To practise the procedure, use a fluorescent dye (such as Texas-Red Dextran) as shown in another video article[13].
3. Tail muscle injection: position anesthetized embryos (1-2 dpf) as for caudal vein injections (see 5.6). Adjust the micromanipulator with the loaded needle to an angle of approximately 65° with respect to the injection plate surface. Inject a bacterial suspension (0.5-1 nL) containing 20- 50 cfu into the muscle above the urogenital opening (**Figure 1D**) without causing damage to the notochord or blood vessels.
4. Otic vesicle injection: position anesthetized embryos (2-3 dpf) as for caudal vein injections (see 5.6). Adjust the micromanipulator with the loaded needle to an angle of approximately 65° with respect to the injection plate surface. Inject approximately 20 bacteria (0.5 nL) into the otic vesicle (**Figure 1E**) with low pressure to avoid local tissue rupture.
5. Notochord injection: line up anesthetized embryos (1-2 dpf) on a flat 1% agarose injecting plate such that the embryos are positioned with their tail pointing away from the needle tip. Insert the needle through the tail muscle tissue into the notochord (**Figure 1F**) and inject approximately 20-50 bacteria (maximal 0.5 nL). Take care not to inject too much volume to avoid rupture of the notochord.
6. Yolk injection: position eggs containing embryos at the 16 to 1000 cell stage on a 1% agarose injecting plate with rectangular or *V*-shaped channels made with a channel mold [12] or online at http://zfin.org/zf_info/zfbook/chapt5/5.1.html. Pierce the needle through the chorion into the center of the yolk (**Figure 1G**) and inject 20-40 bacteria (1-2 nL). The use of 2% PVP40 carrier solution for the bacterial suspension (see step 3.5) is important to prevent early bacterial spread into the embryo.

7. Stereo Imaging of the Infection

1. Anesthetize the infected embryos in a 1% agarose layered Petri dish covered with egg water containing Tricaine (see 4.5).
2. Align the embryos in the correct position for imaging under a fluorescence stereo microscope with a hair loop tool (**Figure 2**). Before the swim bladder has inflated (1-5 dpf), the embryos will lie flat on their sides enabling lateral view imaging.
3. If a different position than the lateral view is required, mount the embryos in 1.5% methyl cellulose. Manipulate the embryo into the required position with a hair loop tool (**Figure 2**).
4. Return the embryos to egg water after imaging.

8. Confocal Imaging of the Infection

1. Place a drop of Low Melting Point agarose (Lonza Inc., 1.5% (w/v) in egg water) on the glass bottom of a WillCo-dish (WillCo Wells, GWSt-5040) if an inverted confocal microscope is used, or on a single cavity depression slide (Agar Scientific, L4090) if an upright confocal microscope is used.
2. Place the anesthetized embryo into the agarose drop with limited amount of egg water and manipulate the embryo into position with a hair loop tool (**Figure 2**). When using an inverted microscope it is important that the embryo's region of interest is flat against the glass bottom of the imaging dish. An upright microscope can be used in combination with water immersion or long distance dry objectives and the embryo should be positioned such that the region of interest is as close to the objective as possible.
3. Let the agarose solidify and submerge the agarose drop in egg water containing Tricaine (see 4.5). If an upright microscope is used, place a glass cover slip on top of the depression cavity (do not allow air bubbles to form).
4. Sequentially acquire fluorescence and transmission images.
5. Carefully remove the agarose from the embryo with fine tweezers and place the embryo back into egg water if it is required for further experiments.

9. Representative Results

Injection of *Salmonella typhimurium* or *Mycobacterium marinum* bacteria into the blood island of embryos at 1 dpf results in the rapid phagocytosis by macrophages. The *mpeg1* gene has recently been identified as a faithful marker of embryonic macrophages, colocalizing with the well-established macrophage marker *csf1r* (*fms*) and not overlapping with neutrophil markers such as *mpx* (*mpo*) and *lyz* [4, 14] . For live

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imaging of bacterial phagocytosis we used transgenic lines in which the *mpeg1* promoter drives fluorescent protein expression in macrophages [14]. These transgenic lines either have the *mpeg1* promoter fused directly to the *gfp* gene, or employ a two-component system where the *mpeg1* promoter drives expression of the yeast Gal4 transcription factor that activates a second transgene with the Gal4 recognition sequence (UAS, upstream activating sequence) fused to the *kaede* gene. When the blood island injection (protocol 5; **Figure 1A**) is performed correctly, the bacteria will immediately flow through the blood circulation and spread throughout the embryo. Dissemination of the relatively large and brightly fluorescent Ds-RED labeled *S. typhimurium* bacteria can be imaged directly with a stereo fluorescence microscope (**Figure 3A**), and confocal imaging at 2 hpi shows that many bacteria are phagocytosed by fluorescent macrophages (**Figure 3B-C**). The injection of as little as 25 cfu of wild type *S. typhimurium* bacteria will result in a lethal infection, while a similar dose of bacteria of an avirulent strain, such as Ra, can be cleared by the embryonic immune system [3]. An injection dose of 250 cfu was used to determine transcriptional responses to *S. typhimurium* infection in the zebrafish embryo and demonstrated the induction of a strong pro-inflammatory gene expression response [15]. In contrast, the intravenous injection of *M. marinum* bacteria does not elicit a strong pro-inflammatory response, but leads to a persistent infection where infected macrophages form tight aggregates that are considered as the initial stages of granulomas, which are the hallmark of tuberculosis [2]. Confocal imaging of such a granuloma-like aggregate in the *Tg*(*mpeg1:EGFP)gl22* line[14] at 5 dpi shows the intracellular growth of mCherry-labeled *M. marinum* bacteria inside the green fluorescent macrophages (**Figure 3D-E**).

Other routes of infection are useful for different purposes. Bacteria can be injected into the hindbrain ventricle at 32 hpf (**Figure 1C)**, which is a compartment devoid of macrophages. Injection of 20-100 mCherry-labeled *M. marinum* bacteria into this compartment leads to the rapid infiltration by macrophages that phagocytose the bacteria (**Figure 4A**). Another method to study the directed migration of innate immune cells is injection of bacteria into the tail muscle (**Figure 1D**). However, tail muscle injections also cause tissue damage that by itself elicits some attraction of leukocytes. Such a wounding response can be avoided when carefully injecting a small volume (0.5-1 nL) into the otic vesicle (**Figure 1E**). As shown here by using the *Tg(mpx:EGFP)i114* line[16], injection of approximately 20 cfu of *S. typhimurium* into the otic vesicle leads to the attraction of neutrophils at 3 hpi (**Figure 4D**), while this response is not observed in PBS control injections (**Figure 4E**). The notochord, which appears to be resistant to infiltration by leukocytes, is a permissive compartment for the growth of *M. marinum* mutants that are strongly attenuated when injected in other tissues [7]; (**Figure 4F**). Finally, early injection of *M. marinum* into the yolk of embryos provides an alternative method to achieve a systemic infection. We generally perform these injections around the 16 cell stage (**Figure 1G**), but bacterial injections into the yolk can also be performed at later stages (up to the 1000 cell stage), or earlier (1 to 8 cell stage) for co-injections with morpholinos [8]. Following yolk injection of a dose of 20-40 cfu, *M. marinum* bacteria spread over several days into the embryonic tissues and form granuloma-like aggregates similar to those observed upon the conventional intravenous injection method [8]; (**Figure 4G**). The yolk injection method is not suitable for *S. typhimurium* infection, because its rapid growth in the yolk causes early lethality. The yolk infection method for *M. marinum* infection will be useful for high-throughput applications since it can be automated using an injection robot [8].

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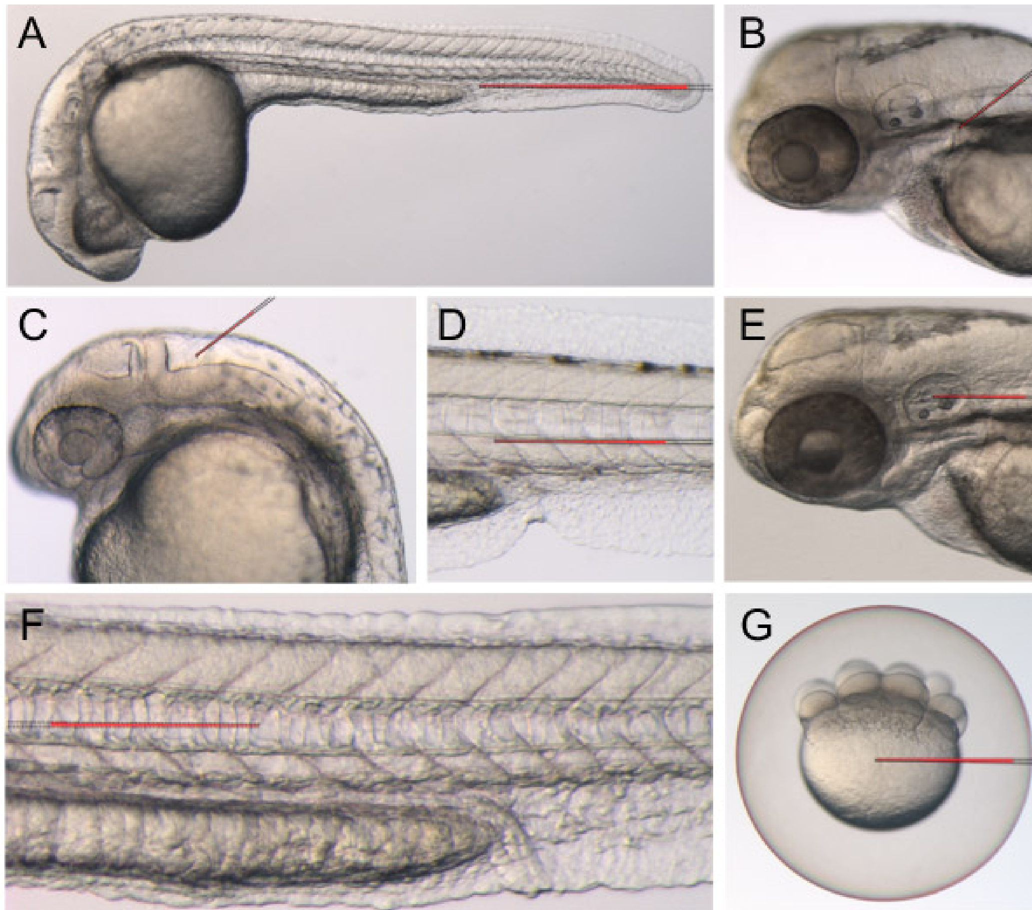

Figure 1. Overview of injection methods used for establishing systemic or local infections in zebrafish embryos. (A-B) Intravenous injections for establishing a rapid systemic infection are performed into the caudal vein at the posterior blood island at 1 dpf (A) or into the Duct of Cuvier at 2-3 dpf (B). (C-E) Local injections for studying macrophage and neutrophil chemotaxis are performed into the hindbrain ventricle at 1 dpf (C), the tail muscle at 1-2 dpf (D) or the otic vesicle at 2-3 dpf (E). (F) Injections to create an infection apparently inaccessible to phagocytes are performed into the notochord at 1-2 dpf. (G) Injections to create an early systemic infection with slow growing bacteria such as *M. marinum* can be performed into the yolk at the 16-1000 cell stage. All images were taken with a Leica M165C, PLANAPO 1.0x connected to a Leica DFC420 camera (Leica 10446307 0.8x).

Figure 2. Hair loop tool. A piece of human hair is inserted as a loop into the opening of a Pasteur pipette and fixed in place with super glue or with Tipp-Ex. This provides a convenient tool for gently manipulating fragile zebrafish embryos.

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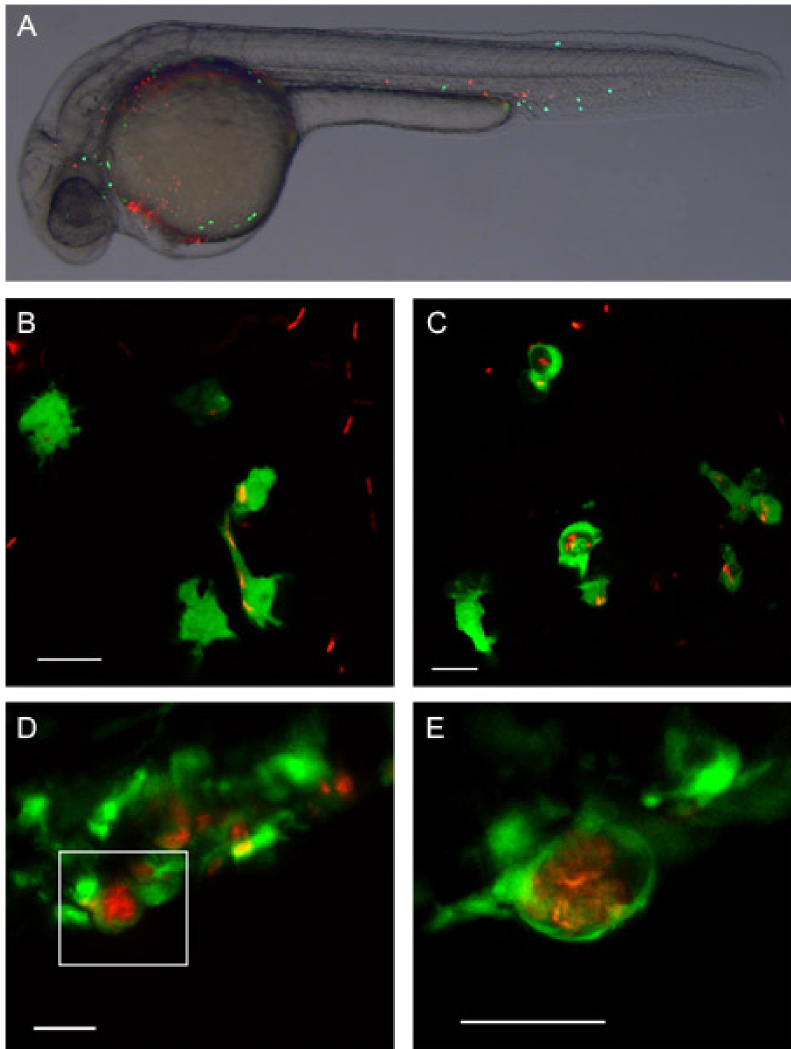

Figure 3. Intravenous injections of red fluorescent *Salmonella typhimurium* and *Mycobacterium marinum*. Ds-RED-labeled *S. typhimurium* SL1027 bacteria (A-C) and mCherry- labeled *M. marinum* Mma20 bacteria (D-E) were injected into the blood island of *Tg(mpeg1:Gal4-VP16)gl24;Tg(UAS-E1b:Kaede)s1999t* (A-C) or *Tg(mpeg1:EGFP)gl22* (D-E) zebrafish embryos at 28 hpf. (A) Stereo-fluorescence and bright-field overlay image showing dissemination of *S. typhimurium* in the blood circulation at 2 hpi (Leica MZ16FA microscope with Leica DFC420C camera). (B-C) Confocal z-stack projections showing red *S. typhimurium* bacteria phagocytosed by green macrophages at 2 hpi (Leica TCS SPE, HCX APO objective 40x 0.8 NA). Bacteria that are still extracellular can also be observed. (D) Confocal z-stack projection showing a granuloma-like aggregate containing *M. marinum* Mma20-infected and uninfected macrophages at 5 dpi (Leica TCS SPE, HCX APO 40x 0.8 NA). Macrophages in green and bacteria in red. (E) Confocal z-stack projection of an individual macrophage (green) with intracellular *M. marinum* bacteria (red). The area depicted in D by the white rectangle was imaged with a higher magnification objective (Leica TCS SPE, HCX PL APO 63x 1.2 NA). Scalebars: 20 µm.

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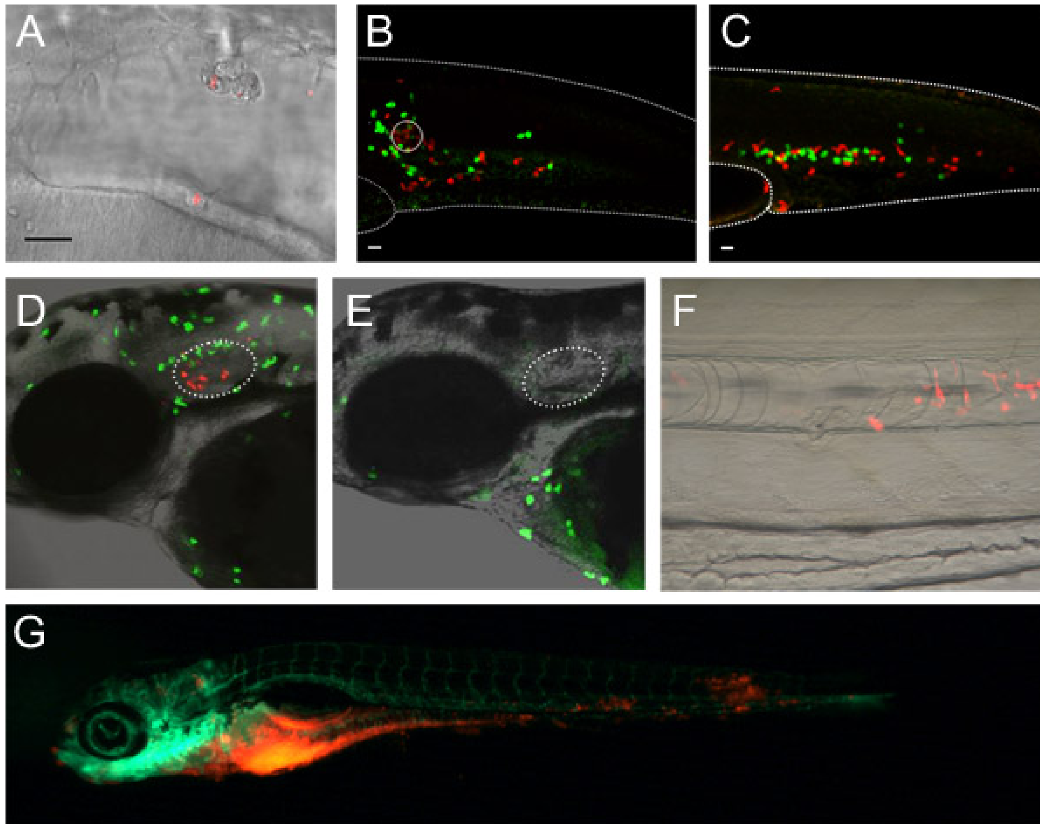

Figure 4. Alternative routes for infection of zebrafish embryos. (A) mCherry-labeled *M. marinum* Mma20 bacteria were injected into the hindbrain ventricle at 32 hpf. Fluorescence and transmission overlay image showing mCherry-labeled bacteria phagocytosed by macrophages at 5 hpi (Leica TCS SPE, HCX PL FLUO TAR 40.0x 0.7 NA). (B) *S. typhimurium* was injected into the tail muscle at 1 dpf. Attraction of myeloid cells to the injection site (white circle) is shown at 3 hpi by fluorescent in situ hybridization [4], (C) whereas in uninjected embryos there are normally no myeloid cells at this morphological site. Although the embryos do not contain mature neutrophils at this stage, two populations of myeloid cells can be distinguished, one expressing the macrophage marker *mfap4* (red) and one expressing the neutrophil marker *mpx* (green) (Leica TCS SPE, HC PL FLUOTAR 10.0x 0.3 NA). Fluorescence of the bacteria is lost after the in situ hybridization procedure. (D) Ds-RED-labeled *S. typhimurium* bacteria were injected into the otic vesicle of *Tg(mpx:EGFP)i114* zebrafish at 2 dpf. Stereo-fluorescence and bright-field overlay images show that *mpx:EGFP* labeled neutrophils cells are attracted to the infected otic vesicle (dotted ellipse) at 3 hpi, (E) whereas control injection of PBS into the otic vesicle of *Tg(mpx:EGFP)i114* zebrafish does not show attraction of neutrophils to the uninfected otic vesicle (dotted ellipse) (Leica MZ16FA with Leica DFC420C camera). (F) mCherry-labeled *M. marinum* bacteria of the attenuated E11 *eccCb1*::tn mutant strain [17] were injected into the notochord at 1 dpf. Proliferation inside the notochord was imaged at 5 dpi (Leica MZ16FA microscope with DC500 camera). (G) mCherry-labeled *M. marinum* E11 bacteria were injected into the yolk of 16-cell stage zebrafish embryos of the *Tg(fli1a:EGFP)* line that expresses *gfp* in endothelial cells of blood and lymph vessels. Formation of granuloma-like aggregates of infected cells in the tail region is observed at 5 dpi (Leica MZ16FA microscope with Leica DFC420C camera; image from [8]).

Discussion

The infection methods described in this article are frequently used to study the function of host innate immunity genes or bacterial virulence genes[1]. The intravenous micro-injection methods (protocols 5 and 6.1) are used most often for such studies. The caudal vein at the posterior blood island is the most convenient location for intravenous injection of 1-day-old embryos. As the caudal vein becomes more difficult to penetrate at later stages, we prefer the Duct of Cuvier as intravenous injection site for embryos at 2-3 dpf. In all cases it is critical to inject the embryos with consistent numbers of bacteria, which should be checked by plating injection inocula for cfu counting. The following aspects must be considered to achieve reproducible injections. Firstly, it is essential to have high-quality glass microcapillary injection needles. If the needle tip is too large, the injection will create a puncture hole large enough for bleeding to occur and the injected bacteria will flow out of the blood circulation. Secondly, bacteria must be injected directly into the blood circulation. If the needle tip is not inside the vein or if injection fluid spreads into the yolk sac extension, the embryo must be discarded from the experiment. Thirdly, using PVP40 as a carrier for injecting *M. marinum* will assist in preventing the bacteria from sinking in the glass needle. PVP40 improves the homogeneity of the suspension, resulting in more reproducible inocula throughout the duration of injections. PVP40 may also be used for *S. typhimurium* injections, but here it is less important because the larger size and bright fluorescence of the bacteria allows a visual control over the injection inoculum during experiments. For practising the injections we recommend the use of phenol red dye (1% v/v) or 1 µm fluorescent spheres available in different colors (Invitrogen). Once the technique is mastered, it takes approximately 30 minutes to inject 50-100 embryos, including the time required for aligning the embryos on agarose plates and adjusting the bacterial injection volume.

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While intravenous injections into the blood island (protocol 5) or into the duct of Cuvier (protocol 6.1) are most commonly used, the alternative routes of infection described in this video article have proved useful to study macrophage and neutrophil chemotaxis (protocols 6.2, 6.3, and 6.4), to study growth of attenuated bacterial strains (protocol 6.5), and to adapt the zebrafish infection model for high-throughput applications (protocol 6.6) [4-8]. The described micro-injection methods can also be applied for injections of viruses [18, 19], fungal spores [14, 20] or protozoan parasites (Maria Forlenza, personal communication). A useful addition to the injection procedures described in this video article is a recently described procedure for subcutaneous injection of bacteria into zebrafish embryos [21]. This procedure was applied to study phagocytosis of bacteria by neutrophils, which were found to efficiently engulf bacteria on tissue surfaces but, in contrast to macrophages, were virtually unable to phagocytose bacteria upon injection into the blood or into fluid-filled body cavities. For subcutaneous injections embryos are positioned similar as for the tail muscle injections described in this video article, but the needle is inserted just under the skin to inject the bacteria over a somite.

It is important to consider that micro-injection is essentially an artificial route of infection. However, early embryos are highly resistant to external exposure to bacterial pathogens. In fact, immersion assays, where embryos are bathed in a bacterial suspension, are not reproducible in our hands [22]. While some embryos may become infected upon immersion, we have observed a large variation in mortality rates and in the expression of inflammatory marker genes between individual embryos in such assays. The micro-injection methods described in this article achieve reproducible infections and are particularly useful to study bacterial interactions with host innate immune cells. An efficient approach to quantify bacterial infection in individual embryos is to analyze the fluorescent images of infected embryos with custom-made, dedicated pixel quantification software [17, 23]. This approach has been shown to correlate well with results of cfu counts after plating of infected embryos. Similarly, relative changes in leukocyte numbers per embryo have been quantified by digital image analysis in transgenic leukocyte fluorophore reporter lines; this approach would be applicable to quantifying the host leukopoietic response to infection[24].

The function of host innate immune genes can be investigated efficiently *in vivo* by combining the described infection models with morpholino knockdown. Morpholinos are synthetic antisense oligonucleotides that target specific RNAs and reduce the expression of the gene products when injected into 1-cell stage zebrafish embryos as shown in other video articles in this journal [10, 25]. In morpholino knockdown studies, it is of great importance that all embryo groups to be compared are staged correctly prior to bacterial injections. This is especially important because embryos have a developing immune system that becomes increasingly competent over time.

There are several transgenic reporter lines currently available to distinguish the major innate immune subsets, macrophages and neutrophils, in zebrafish embryos. The *mpx* and *lyz* promoters have been used to generate neutrophil reporter lines [16, 26, 27], and the *csf1r* (*fms*) and *mpeg1* promoters were used to produce macrophage reporters [14, 28]. While *csf1r* (*fms*) is additionally expressed in xanthophores, *mpeg1* expression is exclusively in macrophages. As shown in this video article, the recently developed *mpeg1* reporter lines are highly useful for imaging bacterial phagocytosis by macrophages.

Disclosures

The authors have nothing to disclose.

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