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Development of a larval zebrafish infection model for Clostridioides difficile

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Dear Editor,

please find enclosed our manuscript entitled “Development of zebrafish infection model for *Clostridoides difficile*” which we would like to submit for publication in the Journal of Visualized Experiments.

Infections with *Clostridium difficile* are among the most common hospital acquired infections world-wide and account for numerous death per year, because in the recent years multi-resistant strains for this infectious pathogen have emerged and are spread around the world. Yet the response of the innate immune system to *Clostridioides difficile* infections (CDI) are understudied, because infections of the intestine occur deep inside the body and innate immune cells are highly motile. Thus a small, nearly transparent vertebrate would serve as a well-suited infection model to allow for monitoring pathogen as well as innate immune cell behaviour directly *in vivo* by high resolution microscopy. Zebrafish larvae contain these advances and are also molecularly tractable to generate transgenic strains with fluorescently labeled macrophages or neutrophils. However, currently available zebrafish reporter strains only display weak fluorescence in innate immune cells. In addition, while protocols for infection of zebrafish with bacteria have been established these are usually suited for aerobic but not anaerobic pathogens such as *Clostridium difficile*.

We have therefore set out to establish a fluorescent labelling method for this Gram-positive pathogen. Furthermore, we provide methods for different infection routes for this bacterial strain either by microinjection or by microgavage through the open mouth of zebrafish larvae to deposit the pathogen directly inside the gastro-intestinal tract. In addition, we provide experimental approaches for confirming successful infection of the larval zebrafish intestinal tract. For this we demonstrate protocols for recovering administered bacteria either by direct culture or by culturing bacteria derived from spore formation. Furthermore, we demonstrate differences in pathogen survival when gnotobiotic zebrafish are used compared to wild type larvae. In summary, we provide a package of methods suited to investigate bacterial infections of anaerobic pathogens and resulting innate immune cell response behavior in transparent zebrafish larvae. We are convinced that these technical advancements will be of help for infection biologists and zebrafish researchers alike and therefore hope for your positive approval for publishing this manuscript in the Journal of Visualized Experiments.

Yours sincerely,

R. Köster

(Reinhard Köster)

TITLE:

Development of a Larval Zebrafish Infection Model for *Clostridioides difficile*

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KEYWORDS:

zebrafish, *Danio rerio, Clostridioides difficile* infection, microinjection, live staining, microgavage, anaerobe, dissection, gnotobiotic zebrafish

SUMMARY:

Presented here is a safe and effective method to infect zebrafish larvae with fluorescently labeled anaerobic *C. difficile* by microinjection and noninvasive microgavage.

ABSTRACT:

Clostridioides difficile infection (CDI) is considered to be one of the most common healthcare-associated gastrointestinal infections in the United States. The innate immune response against *C. difficile* has been described, but the exact roles of neutrophils and macrophages in CDI are less understood. In the current study, *Danio rerio* (zebrafish) larvae are used to establish a *C. difficile* infection model for imaging the behavior and cooperation of these innate immune cells in vivo. To monitor *C. difficile*, a labeling protocol using a fluorescent dye has been established. A localized infection is achieved by microinjecting labeled *C. difficile,* which actively grows in the zebrafish intestinal tract and mimics the intestinal epithelial damage in CDI. However, this direct infection protocol is invasive and causes microscopic wounds, which can affect experimental results. Hence, a more noninvasive microgavage protocol is described here. The method involves delivery of *C. difficile* cells directly into the intestine of zebrafish larvae by intubation through the open mouth. This infection method closely mimics the natural infection route of *C. difficile*.

INTRODUCTION:

C. difficile is a gram-positive, spore-forming, anaerobic, and toxin-producing bacillus that is the leading cause of severe infections in the gastrointestinal tract[1]. Typical symptoms of CDI include diarrhea, abdominal pain, and fatal pseudomembranous colitis, and it can sometimes lead to death[1,2]. Evidence has shown that host immune responses play a critical role in both the progression and outcome of this disease[3]. In addition to the immune response, the indigenous gut microbiota is crucial for the onset and pathogenesis of CDI[4]. In the past decade, both the number of cases and the mortality rate of CDI have increased significantly due to the emergence of a hypervirulent strain of *C. difficile* (BI/NAP1/027)[5,6]. A better understanding of underlying immune mechanisms and the role of microbiota during CDI will help lead to new therapeutic developments and advances, enabling better control of this epidemic.

Several animal models, such as the hamster and mouse, have been developed to provide insight into the immune defense against *C. difficile*[7,8]. However, the role of innate immune cells is still poorly understood, particularly since innate immune cell behavior is mainly derived from histological analysis or cultured cells in vitro. Therefore, establishing a transparent zebrafish model to reveal the innate immune response to *C. difficile* inside of a living vertebrate organism will facilitate such studies. Zebrafish larvae have a functional innate immune system, but they lack the adaptive immune system until 4–6 weeks after fertilization[9]. This unique feature makes zebrafish larvae an excellent model to study the isolated response and function of innate immune cells in CDI.

This report describes new methods using zebrafish larvae to study the interactions between *C. difficile* and innate immune cells, such as macrophages and neutrophils. First, a localized microinjection protocol that includes *C. difficile* inoculum and staining is presented. Using in vivo confocal time-lapse imaging, the response of neutrophils and macrophages towards the infection site is recorded, and the phagocytosis of bacteria by neutrophils and macrophages is observed. However, it has been reported that the injection itself causes tissue damage and leads to the recruitment of leukocytes independent of the bacteria[10]. Therefore, a noninvasive microgavage protocol to deliver *C. difficile* into the intestine of zebrafish larvae is subsequently described. Previous studies have demonstrated that indigenous gastrointestinal microbiota protect a host against the colonization of *C. difficile*[11]. Therefore, gnotobiotic zebrafish larvae are also used to predispose the zebrafish that are infected [12]. Afterwards, intestinal dissection is performed to recover viable *C. difficile* and validate the duration of their presence in zebrafish intestinal tracts.

PROTOCOL:

All animal work described here was performed in accordance with legal regulations (EU-Directive 2010/63, license AZ 325.1.53/56.1-TUBS and license AZ 33.9-42502-04-14/1418).

1. Preparation of low melting agarose, gel plate, and microinjection/microgavage needles

1.1. Dissolve 0.08 g of low melting agarose (**Table of Materials**, agarose A2576) in 10 mL of 30% Danieau's's medium (0.12 mM $MgSO_4$, 0.18 mM Ca $[NO_3]_2$), 0.21 mM KCl, 1.5 mM HEPES (pH = 7.2), and 17.4 mM NaCl, stored at room temperature (RT) to obtain a 0.8% solution.

NOTE: Higher or lower concentrations of agarose can be used. However, the required time to solidify varies for different brands of agarose, even at the same concentration.

1.2. Prepare microinjection and microgavage needles from glass capillaries (**Table of Materials**).

1.2.1. Use a micropipette puller with the following settings (note that units are specific to the puller used here; see **Table of Materials**): microinjection needles (air pressure = 500; heat = 400; pull = 125; velocity = 75; time = 150); and microgavage needles (air pressure = 500; heat = 400; pull = 100; velocity = 75; time = 150). Use a microloader tip to load 3 µL of nuclease-free H_2O into the pulled needle.

1.2.2. Introduce the needle into the injector and fasten it properly. Adjust the needle to a suitable angle for injection. Set the injection pressure between 600–900 hPa for microinjection needle and 200–300 hPa for gavage needle.

1.2.3. Place a drop of mineral oil onto the black circle of the calibration slide. Use fine forceps to clip the tip of the needle. Inject one drop into the mineral oil to measure the size of the droplet.

1.2.4. For microinjection, adjust the injection time to obtain a droplet with a diameter of 0.10–0.12 mm, which equals a volume of 0.5–1.0 nL. For microgavage, obtain a droplet with a diameter of 0.18–0.20 mm, which equals a volume of 3–5 nL.

1.3. Prepare a 1.5 % agarose plate with agarose (**Table of Materials**, 8050) in a 10 cm Petri dish in 30% Danieau's's medium using a plastic mold as the microgavage mold. Store at 4 °C to prevent desiccation until needed. Warm to RT or 28 °C prior to the experiment.

2. Preparation and labeling of *C. difficile* and spores with fluorescent dye

2.1. Prepare a 1 mM stock solution of a fluorescent dye (**Table of Materials**). Because the dye is sold in 50 µg aliquots of powder, add 69 µL of DMSO to the vial to obtain a 1 mM stock concentration.

2.2. Prepare a 100 µM working solution of the fluorescent dye by adding 2 µL of 1 mM stock solution to 18 µL of DMSO in a centrifuge tube, and mix well.

2.3. Culture *C. difficile* (R20291, a ribotype 027 strain) by inoculating 10 mL of BHIS liquid medium with two to three colonies from a plate in an anaerobic hood without shaking overnight. BHIS is BHI supplemented with 0.5% (w/v) yeast extract and 0.1% (w/v) L-cysteine. Dissolve 1 g of L-cysteine in 10 mL of ddH_2O and sterilize by filtration, then add to the

autoclaved medium to obtain a final concentration of 1 g/L. Plates are prepared by adding 15 g/L agar-agar to the medium before autoclaving. Selective culturing of *C. difficile* is done by using chromID *C. difficile* plates (**Table of Materials**).

2.4. Staining *C. difficile* with the fluorescent dye

2.4.1. Harvest *C. difficile* at an OD_{600} of 1.0–1.2 and wash 1x with 1 mL of 1x PBS (5000 x *g* for 3 min at RT). Resuspend in 1 mL of 1 x PBS.

2.4.2. Add 3 µL of working solution of the fluorescent dye into 1 mL of bacteria suspension. Incubate the sample for 15 min at RT in the dark. Wash the stained C. *difficile* once with 1x PBS to remove residual dye and resuspend in 1x PBS to an OD_{600} of 1.0 (1.0 OD_{600} is approximately equivalent to 10^8 cfu/mL).

3. Injection of stained *C. difficile* into zebrafish larvae

3.1. Anesthetize 20–30 zebrafish larvae at 5 days post-fertilization (referred to here as 5 dpf) with 0.02%–0.04% tricaine (tricaine powder is dissolved in double-distilled water and adjusted to pH = 7 with 1 M Tris-HCL solution) in 30% Danieau's's medium ~10 min before injection. Transfer the anesthetized larvae to a fresh 10 cm Petri dish and remove any excess 30% Danieau's's medium.

3.2. Place a drop of 0.8% low melting agarose onto the zebrafish larvae to cover. Gently adjust the larvae to a lateral position. Place the Petri dish on ice for 30–60 s to allow the low melting agarose to solidify. Add 30% Danieau's medium containing 0.02%–0.04% tricaine to cover the agarose.

3.3. Prepare the injection solution. Add 1 µL of 0.5% phenol red in PBS solution into 9 µL of the dye-stained *C. difficile* inoculum to visualize the injection process.

3.4. Load a calibrated microinjection needle with the injection solution using a microloader. Mount the loaded needle onto a micromanipulator and position it under a stereomicroscope.

3.5. Adjust the injection pressure between 600–900 hPa. Set the injection time to 0.1–0.3 s to obtain 0.5–1.0 nL. Set the needle in the micromanipulator at a ~45° angle pointing toward the embedded larvae.

3.6. Place the needle tip above the gastrointestinal tract close to the urogenital pore. Pierce through agarose then the muscle with the needle tip, then insert it into the intestinal lumen and inject 0.5–1.0 nL of *C. difficile*. Use a fluorescence microscope to monitor the injected larvae and pick up the properly injected larvae for confocal imaging.

4. Generation of gnotobiotic zebrafish larvae

4.1. Use the well-established natural breeding method to generate gnotobiotic zebrafish embryos, including: in vitro fertilization, washing with antibiotic-containing medium (1 µg/mL amphotericin B, 10 µg/mL kanamycin, and 20 µg/mL ampicillin), washing with 0.1% wt/vol polyvinyl pyrrolidone-iodine (PVP-I) solution, and incubation of the embryos in a cell culture hood[12].

4.2. Maintain all gnotobiotic zebrafish larvae under gnotobiotic conditions until 5 dpf or just before the gavage. After the gavage, zebrafish larvae will be transferred into a standard incubator but with sterile 30% Danieau's medium.

5. Gavage of zebrafish larvae

5.1. Calibrate the microgavage needle as described in step 1.2.

5.2. Measure the diameter of the tip of the needle by placing the needle on a calibration slide with one drop of mineral oil. Ensure that the tip is 30–40 µm in diameter, blunt, and smooth. Discard the sharp or rough needles.

NOTE: Sharp edges of needles can be blunted by quick flaming.

5.3. Prepare the gavage solution as described in step 3.3.

5.4. Load and mount the needle onto a micromanipulator as described in step 3.4. Adjust the micromanipulator to position the needle at a 45° angle.

5.5. Anesthetize the zebrafish larvae referred to in step 3.1. When the larvae stop moving, transfer them to the groove of a microgavage mold using a Pasteur pipette.

5.6. Place a drop of 0.8% low melting agarose onto the zebrafish larvae to cover. Gently adjust the larvae with heads facing upright at 45° angles in the groove and tails against the wall of the groove. Ensure that the angles of the heads are approximately the same so that they are aligned with the angle of the gavage needles. Place the microgavage mold on ice for 30–60 s, allowing the low melting agarose to solidify in order to stabilize the positions of the larvae.

5.7. Adjust the injection pressure between 200–300 hPa. Set the injection time to 0.1–0.3 s to obtain an injection volume of 3–5 nL of *C. difficile.*

5.8. Gently operate the needle through the agarose then into the mouth of zebrafish larvae, through the esophagus. Once the tip of the needle is inside the anterior intestinal bulb, press the injection pedal to release the bacteria. Fill the lumen of the intestine with the delivered volume. Do not let it overflow from the esophagus or cloaca. Gently withdraw the needle from the mouth of the zebrafish.

5.9. Following gavage, rescue the infected zebrafish larvae from the agarose with a flexible

microloader tip by first cutting the agarose away then by lifting the larvae. Transfer these larvae into sterile 30% Danieau's medium. Rinse the larvae in sterile medium 2x. Transfer the larvae to a fresh 10 cm Petri dish. The larvae will be maintained for up to 11 dpf.

6. Confocal microscopy analysis of injected zebrafish larvae

6.1. Anesthetize zebrafish larvae referred to step 3.1. Make a hole in the bottom of a 35 mm Petri dish with a glass slide attached to the hole, referred as the imaging chamber. Transfer embryos to the bottom of the imaging chamber with an adequate amount of 30% Danieau's medium.

6.2. Add 200–300 µL of 1% low melting agarose to cover the anesthetized larvae. Since an inverted confocal microscope is used, place the infected region of the larvae against the glass slide as closely as possible.

6.3. Let the agarose solidify on ice for 30–60 s. Submerge the agarose with 30% Danieau's containing 0.02%–0.04% tricaine.

6.4. Image the larvae with a confocal laser scanning microscope (**Table of Materials**).

7. Dissection of larval zebrafish intestine to recover viable *C. difficile*

7.1. Isolate gastrointestinal tracts from larvae to analyze bacterial load. Start by euthanizing zebrafish larvae with 0.4 % tricaine.

7.2. Rinse the zebrafish briefly with sterile 1x PBS and transfer them to a fresh agarose plate.

7.3. Dissection of zebrafish

7.3.1. Insert a needle into the dorsal trunk of zebrafish larvae close to the head to immobilize the zebrafish. Remove the head behind the gills with a lancet.

7.3.2. Insert the second needle into the middle of the dorsal trunk. Insert the third needle into the abdomen of the zebrafish and pull the intestine out of the body cavity.

NOTE: Extreme care is needed to isolate the intact intestine. If it is difficult to do so, perform additional pulls to separate the rest of the intestine from the remaining internal organs.

7.3.3. Use a microinjection needle to transfer 10–15 intestines into a 1.5 mL tube containing 200 µL sterile of 1x PBS.

7.3.4. Homogenize the intestines with a pestle to disrupt the tissue and prepare homogenates. Ensure the pestle reaches the bottom of the tube to disrupt all intestines completely.

7.4. Incubate the homogenates in *C. difficile* rearing medium containing D-cycloserine and cefoxitin, with or without taurocholate (TCA, a germinant of *C. difficile* spores) in an anaerobic chamber.

7.5. Incubate the plate anaerobically for 48 h at 37 °C.

7.6. Use bacterial culture for 16S rRNA-PCR.

7.6.1. Resuspend a colony in 50 µL of H_2O and boil it at 95 °C for 15 min. Pellet the lysed debris by centrifugation (14,000 rpm for 2 min at RT) and use 2 µL of the supernatant as a template in 25 µL of PCR-reaction using *C. difficile*-specific primers (Cdiff16Sfw: 5' GTG AGC CAG TAC AGG 3'; Cdiff16Srev: 5' TTA AGG AGA TGT CAT TGG 3').

7.6.2. When bacteria from liquid culture are used, harvest 1 mL of culture and wash 1x with 1 mL of PBS (14,000 rpm for 2 min at RT). Resuspend the pellet in 100 µL of H_2O_{dd} and treat as done above. To further characterize bacterial colonies, streak on a BHIS- or chromID-plate (**Table of Materials**).

REPRESENTATIVE RESULTS:

C. difficile is strictly anaerobic, but the chromophore of fluorescent proteins usually requires oxygen to mature. To overcome this problem, a fluorescent dye was used to stain live *C. difficile* cells that were actively growing (R20291, a ribotype 027 strain; **Figure 1A**). Using the Gal4/UAS system, stable transgenic zebrafish lines were generated for live imaging, in which the *mpeg1.1* or *lyZ* promoters drove the expression of EGFP fluorescent protein in macrophages and neutrophils (respectively) in a Gal4-dependent manner.

The stained *C. difficile* was injected into the zebrafish intestinal tract at 5 dpf, and the infected sites were imaged after 1 h of incubation. Time-lapse imaging showed that both neutrophils and macrophages reached the infection sites (**Figure 1B**), and the number of these two innate immune cells increased until the *C. difficile* was cleared. Clearing occurred by phagocytosis and digestion of the labeled *C. difficile.* **Figure 1C** shows that an activated macrophage phagocytized two *C. difficile* bacteria (see **Supplementary Movie 1**).

[Place **Figure 1** here]

Although microinjection is the most common method to infect zebrafish larvae with pathogens, this method invariably causes tissue damage, which can influence experimental results. To avoid this issue, microgavage was used to deliver fluorescence-labeled *C. difficile* into the intestinal lumen of macrophage and neutrophil reporter lines at 5 dpf, which mimics the natural path of CDI (**Figure 2A**). However, neutrophils and macrophages did not show obvious migration to the gastrointestinal tract for up to 12 h after microgavage (**Figure 2B**). In the meantime, fluorescence of the labeled *C. difficile* disappeared after around 5 h post-microgavage, likely due to either 1) enzymatic destruction, 2) inappropriate pH levels in the intestine of zebrafish, or 3) onset of spore formation and accompanying membraneous changes

in the bacteria (**Figure 2B**). Therefore, an intestinal dissection method was established to detect *C. difficile*.

[Place **Figure 2** here]

To confirm whether *C. difficile* was able to inhabit zebrafish, zebrafish intestines were separated at various timepoints after the gavage. Then, the isolated intestines were homogenized with plastic pestles, and homogenates were incubated in *C. difficile* medium containing D-cycloserine and cefoxitin, with or without TCA. However, actively growing *C. difficile* cells were only detected up to 24 h post-infection both with and without TCA (data not shown). It is speculated that the indigenous microbial communities in conventional larvae prevented *C. difficile* invasion because of their colonization resistance.

Gnotobiotic zebrafish larvae were also used. Intestinal samples 24 hpi were dissected and showed bacterial growth in medium with TCA or without TCA, while no bacteria grew in the control group (**Figure 3A**). However, at later timepoints (i.e., 48 hpi, 72 hpi, and 120 hpi) incubated samples only grew in the medium containing TCA, which suggested that total *C. difficile* in the gut had formed spores (**Figure 3B**). This provides a possible explanation for the loss of fluorescence labeling.

16S rDNA PCR was then used to identify the grown bacteria as *C. difficile*, which produced specific PCR amplicons of predictable size (~800 bp; **Figure 3C**). This result was further verified by sequencing of these PCR products. Afterwards, bacterial cultures were spread on a BHIS plate. Several single colonies were then transferred onto a chromID-plate, which supported only *C. difficile* growth. The bacteria appeared as typical black colonies, which further indicated that bacteria from the zebrafish intestines were *C. difficile* (**Figure 3D**).

[Place **Figure 3** here]

FIGURE LEGENDS:

Figure 1: Staining and infection of *C. difficile* in zebrafish. (**A**) Fluorescently labeled *C. difficile* bacteria within infected zebrafish after microinjection. Scale bar = 5 µm. (**B**) Confocal Z-stack projection showing green fluorescent neutrophils in double transgenic zebrafish *Tg(lyZ: KalTA4)^{bz17}*/Tg(*4xUAS-E1b:EGFP)^{hzm3}* accumulated at the *C. difficile* infection site at 2 h post-infection (hpi). Neutrophils are shown in green and *C. difficile* in red. Scale bar = 50 µm. (**C**) Confocal time-lapse imaging showing GFP-labelled macrophages phagocytosing red fluorescent stained *C. difficile*. Scale bar = 20 µm.

Figure 2: Microgavage of zebrafish larvae with *C. difficile*. (**A**) Representative image of zebrafish larvae at 5 dpf gavaged with fluorescence-stained *C. difficile*. The image was recorded around 3 h post-gavage, showing that *C. difficile* were present in the posterior intestine of zebrafish. Scale bar = 100 µm. (**B**) Confocal time-lapse imaging demonstrating macrophage motility. Double transgenic zebrafish larvae *Tg(mpeg1.1: KalTA4)^{bz16}*/Tg(*4xUAS-E1b:EGFP)^{hzm3}* at

5 dpf were gavaged with fluorescence-labeled *C. difficile*. Confocal time-lapse imaging was performed for up to 12 h. Scale bar = 200 µm.

Figure 3: Detection of *C. difficile* in zebrafish intestine after infection. For each of the independent experiments, 15–20 gnotobiotic zebrafish larvae at 5 dpf were infected with 3–5 nL of 10^8 CFUs/mL *C. difficile* or PBS as a negative control by microgavage. (**A**) The homogenates of dissected intestines were cultured. Bacteria grew in the medium containing TCA 72 h after infection of gnotobiotic zebrafish larvae (1, non-infected control group; 2, R20291-infected zebrafish; n = 3). (**B**) Schematic illustration of overall experiment results after 24 h, 48 h, 72 h, and 120 h post-infection with *C. difficile* (n = 3) (**C**) Bacterial samples were tested by 16S rDNA PCR at 72 h post-microgavage (1, non-infected control group; 2, R20291-infected zebrafish; n = 3). (**D**) The growth of *C. difficile* on chromID-plate; note the black color of *C. difficile*, which is a feature of these plates (n = 3).

DISCUSSION:

The presented methods modify and extend an existing approach to infect zebrafish larvae by performing both injection and microgavage[10,14]. It also demonstrates an approach to study anaerobic pathogens with zebrafish larvae[22]. In addition, the protocol facilitates the analysis of innate immune cell responses in vivo upon CDI and upon colonization of *C. difficile* in zebrafish. The method is reproducible and easy to conduct in a routine laboratory or clinical environment.

To monitor the phagocytosis of *C. difficile* by leukocytes, two stable transgenic zebrafish lines were used (e.g., Tg[*mpeg1.1: KalTA4*]^{bz16}) to visualize macrophages (KalTA4: a zebrafish-optimized Gal4-variant) and Tg(*lyz: KalTA4*)^{bz17} and visualize neutrophils when crossed with the transgenic background of Tg(4xUAS: EGFP)^{hzm3} carriers. Due to the anaerobic environment that is required for the growth of *C. difficile*, genetic tools to trace *C. difficile* are limited. Although a codon-optimized *mCherryOpt* has been reported to label them, *C. difficile* bacteria must be fixed before exhibiting fluorescence prior to its use in live imaging settings[13]. Therefore, a fluorescent dye was used here to stain live *C. difficile* with red fluorescence, which can be combined with the numerous available green fluorescent transgenic zebrafish strains. This method can easily be applied to other intestinal anaerobic bacteria, such as *Bacteroides fragilis* and *Helicobacter hepaticus.* The representative results demonstrate that both neutrophils and macrophages can recognize and phagocytose *C. difficile* in infected zebrafish.

Both immersion and microinjection methods have been regularly used to infect zebrafish[20-22]. Using immersion methods is straightforward, yet this approach makes it difficult to accurately control the invasion time of bacteria into the intestine. Microinjection is the most common method to infect zebrafish embryos with pathogens, but it invariably causes tissue damage. Hence, microgavage was used to mimic the natural infection route.

However, it was found that dye-stained *C. difficile* became undetectable around 5 h post-microgavage. The reasons for this are currently unclear but may be related to either the 1) intolerance of the fluorophore under intestinal conditions or 2) initiation of spore formation of

C. difficile cells. It was further found that *C. difficile* were not detectable in the culture of whole-larvae homogenates. Therefore, the gut of each infected zebrafish was dissected then cultured to determine whether *C. difficile* still inhabited the intestinal tissue of zebrafish.

Because of the indigenous microbiota of conventional mice, only mice pretreated with an antibiotic cocktail or gnotobiotic mice are susceptible to *C. difficile*[16]. Likewise, it was found that *C. difficile* was only detected in the intestines of gnotobiotic zebrafish but not in conventional wild-type zebrafish 24 h post-infection. Interestingly, intestinal samples of 48 h, 72 h, and 120 h post-infection zebrafish only grew in media containing TCA. As described above, TCA stimulates *C. difficile* spore germination in vitro. This result suggests that active *C. difficile* cells already formed vegetative spores in the zebrafish intestine, and spore-derived colonies were detected using the microgavage approach.

Intriguingly, germ-free zebrafish still did not present any symptoms of CDI, such as intestinal neutrophil influx or even death of zebrafish. This shows that infection by injection may activate innate immune cells by wounding and that only activated macrophages and neutrophils are able to quickly detect *C. difficile*. In addition, a likely explanation for the lack of prominent CDI in zebrafish is based on the structural differences between zebrafish and mammalian intestines[17]. The zebrafish gut lacks intestinal crypts, where *C. difficile* are frequently located[18]. Along with the lower maintenance temperature of zebrafish compared to that in mammals, it was inferred that the onset of CDI in zebrafish does not occur as fast as in mammalian models. However, two anaerobic bacteria of the human microbiota, *Lactobacillus paracasei and Eubacterium limosum,* have been proven to grow inside the zebrafish gut[19]. The technical advances presented here will encourage applications of this method to studying *C. difficile* and other bacteria or pathogens derived from the mammalian gut in molecularly tractable zebrafish larvae in vivo.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Rupnik, M., Wilcox, M. H., Gerding, D. N. Clostridium difficile infection: new developments in epidemiology and pathogenesis. *Nature Reviews Microbiology*. **7**, 526–36 (2009).

2 Yang, Z. et al. Mechanisms of protection against Clostridium difficile infection by the monoclonal antitoxin antibodies actoxumab and bezlotoxumab. *Infection and Immunity*. **83**, 822–31 (2015).

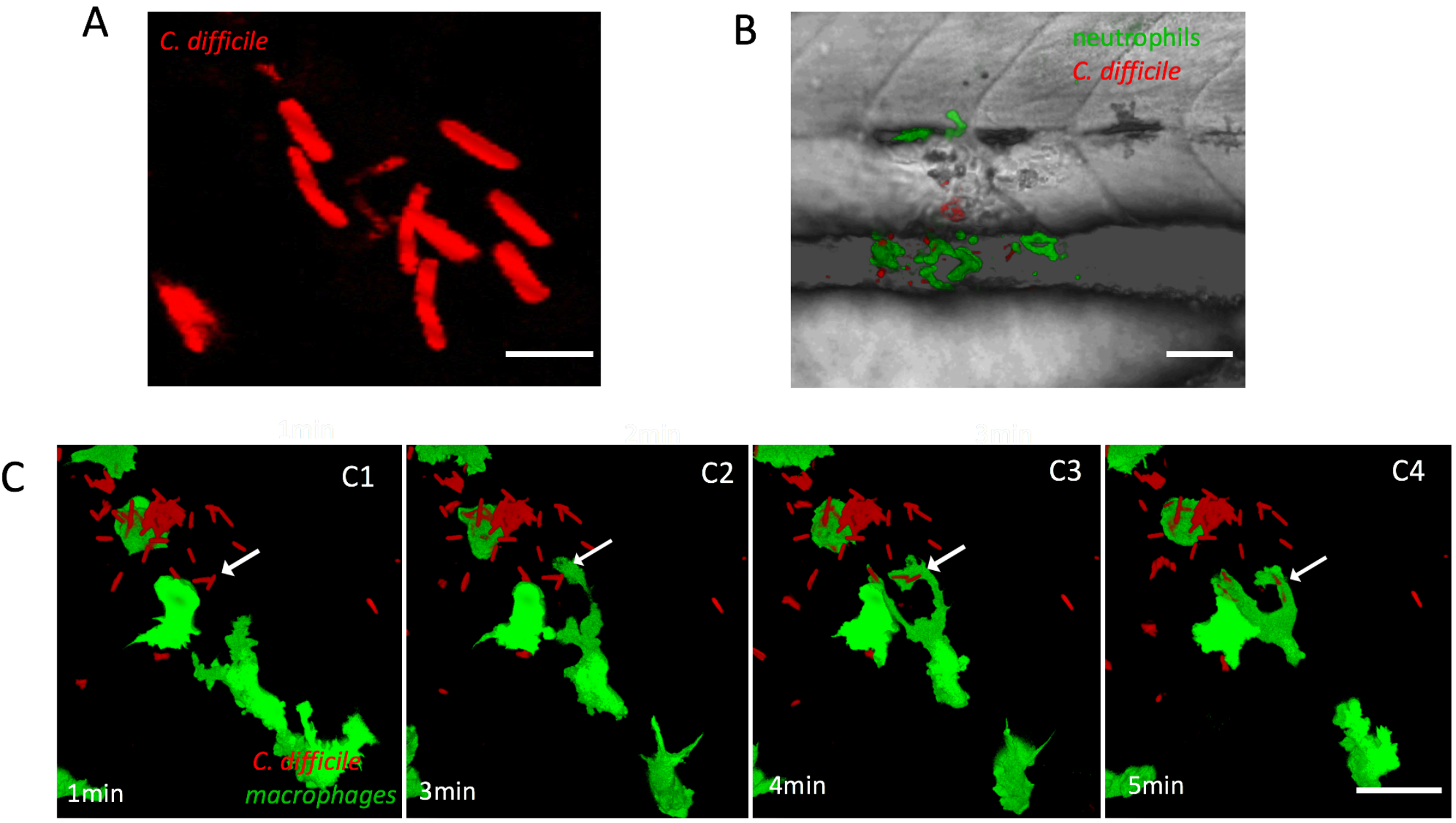
3 Kelly, C. P., Kyne, L. The host immune response to Clostridium difficile. *Journal of Medical*

Microbiology. **60**, 1070–9 (2011).
4 Britton, R. A., Young, V. B. Interaction between the intestinal microbiota and host in Clostridium difficile colonization resistance. *Trends in Microbiology*. **20**, 313–9 (2012).
5 Goorhuis, A. et al. Emergence of *Clostridium difficile* Infection Due to a New Hypervirulent Strain, Polymerase Chain Reaction Ribotype 078. *Clinical Infectious Diseases*. **47**, 1162–70 (2008).
6 Pépin, J. et al. Emergence of fluoroquinolones as the predominant risk factor for Clostridium difficile-associated diarrhea: a cohort study during an epidemic in Quebec. *Clinical Infectious Diseases*. **41**, 1254–60 (2005).
7 Merrigan, M. M., Sambol, S. P., Johnson, S., Gerding, D. N. Prevention of Fatal *Clostridium difficile* –Associated Disease during Continuous Administration of Clindamycin in Hamsters. *The Journal of Infectious Diseases*. **188**, 1922–7 (2003).
8 Chen, X. et al. A Mouse Model of Clostridium difficile-Associated Disease. *Gastroenterology*. **135**, 1984–92 (2008).
9 Page, D. M. et al. An evolutionarily conserved program of B-cell development and activation in zebrafish. *Blood*. **122**, 1–12 (2014).
10 Benard, E. L. et al. Infection of Zebrafish Embryos with Intracellular Bacterial Pathogens. *Journal of Visualized Experiments*. 1–8 (2012).
11 Theriot, C. M., Young, V. B. Interactions Between the Gastrointestinal Microbiome and *Clostridium difficile*. *Annual Review of Microbiology*. **69**, 445–61 (2015).
12 Pham, L. N., Kanther, M., Semova, I., Rawls, J. F. Methods for generating and colonizing gnotobiotic zebrafish. *Nature Protocols*. **3**, 1862–75 (2008).
13 Ransom, E. M., Ellermeier, C. D., Weiss, D. S. Use of mCherry red fluorescent protein for studies of protein localization and gene expression in Clostridium difficile. *Applied and Environmental Microbiology*. **81** (5), 1652–1660 (2015).
14 Cocchiaro, J. L., Rawls, J. F. Microgavage of Zebrafish Larvae. *Journal of Visualized Experiments*. **e4434** (2013).
15 Chen, X. et al. A Mouse Model of Clostridium difficile-Associated Disease. *Gastroenterology*. **135** (6), 1984–1992 (2008).
16 Hutton, M. L., Mackin, K. E., Chakravorty, A., Lyras, D. Small animal models for the study of Clostridium difficile disease pathogenesis. *FEMS Microbiology Letters*. **352**, 140–9 (2014).
17 Brugman, S. The zebrafish as a model to study intestinal inflammation. *Developmental & Comparative Immunology*. **64**, 82–92 (2016).
18 Goulding, D. et al. Distinctive profiles of infection and pathology in hamsters infected with Clostridium difficile strains 630 and B1. *Infection and Immunity*. **77**, 5478–85 (2009).
19 Toh, M. C. et al. Colonizing the Embryonic Zebrafish Gut with Anaerobic Bacteria Derived from the Human Gastrointestinal Tract. *Zebrafish*. **10**, 194–8 (2013).
20 Bloemberg, G. V. et al. Comparison of static immersion and intravenous injection systems for exposure of zebrafish embryos to the natural pathogen Edwardsiella tarda. *BMC Immunology*. **12**, 58 (2011).
21 Díaz-Pascual, F., Ortíz-Severín, J., Varas, M. A., Allende, M. L., Chávez, F. P. In vivo host-pathogen interaction as revealed by global proteomic profiling of zebrafish larvae. *Frontiers in Cellular and Infection Microbiology*. **7**, 1–11 (2017).

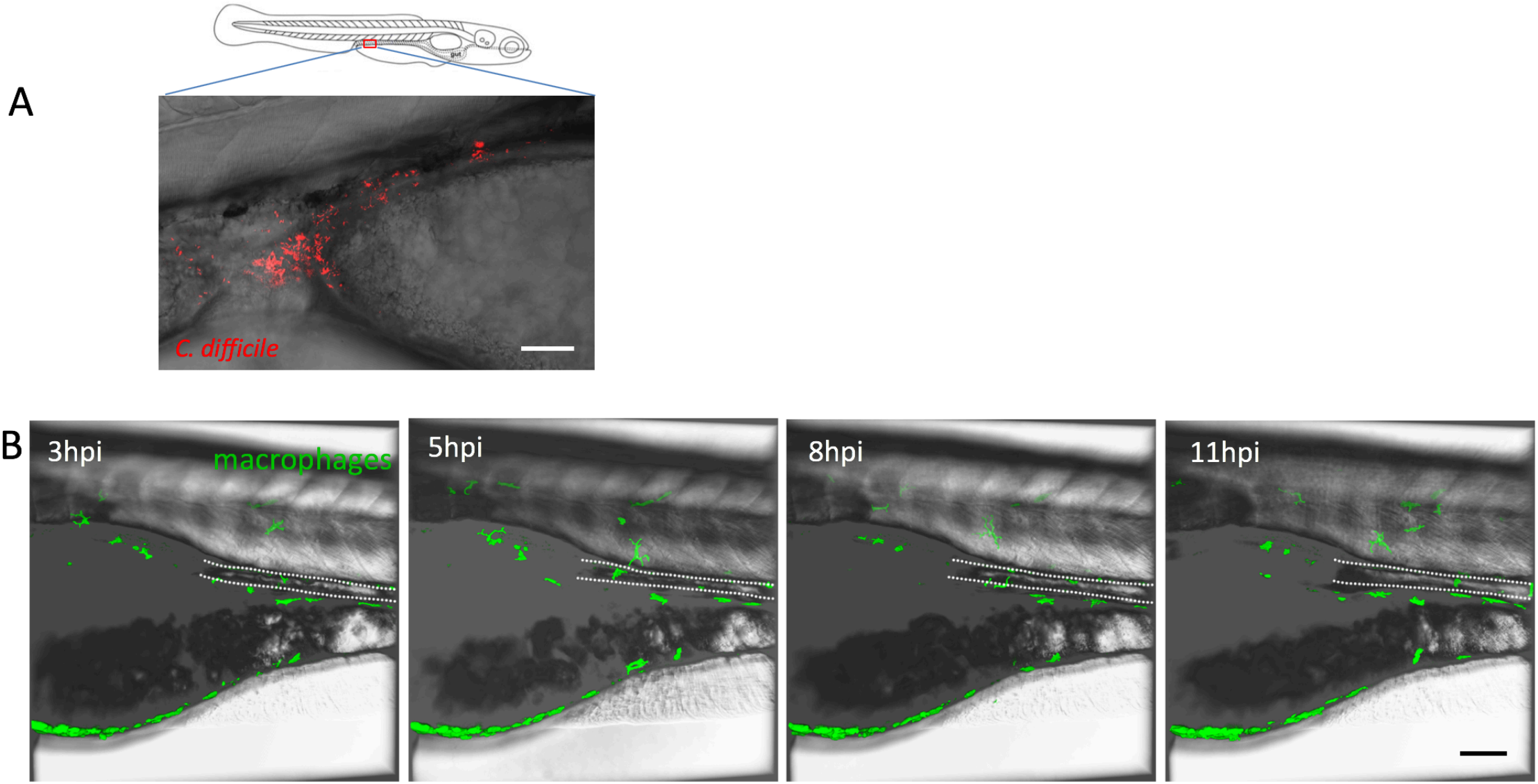
22 Valenzuela, M. J. et al. Evaluating the capacity of human gut microorganisms to colonize the zebrafish larvae (Danio rerio). *Frontiers in Microbiology*. **9**, (2018).

Figure

Click here to access/download;Figure;Figure 1.pdf



Figure



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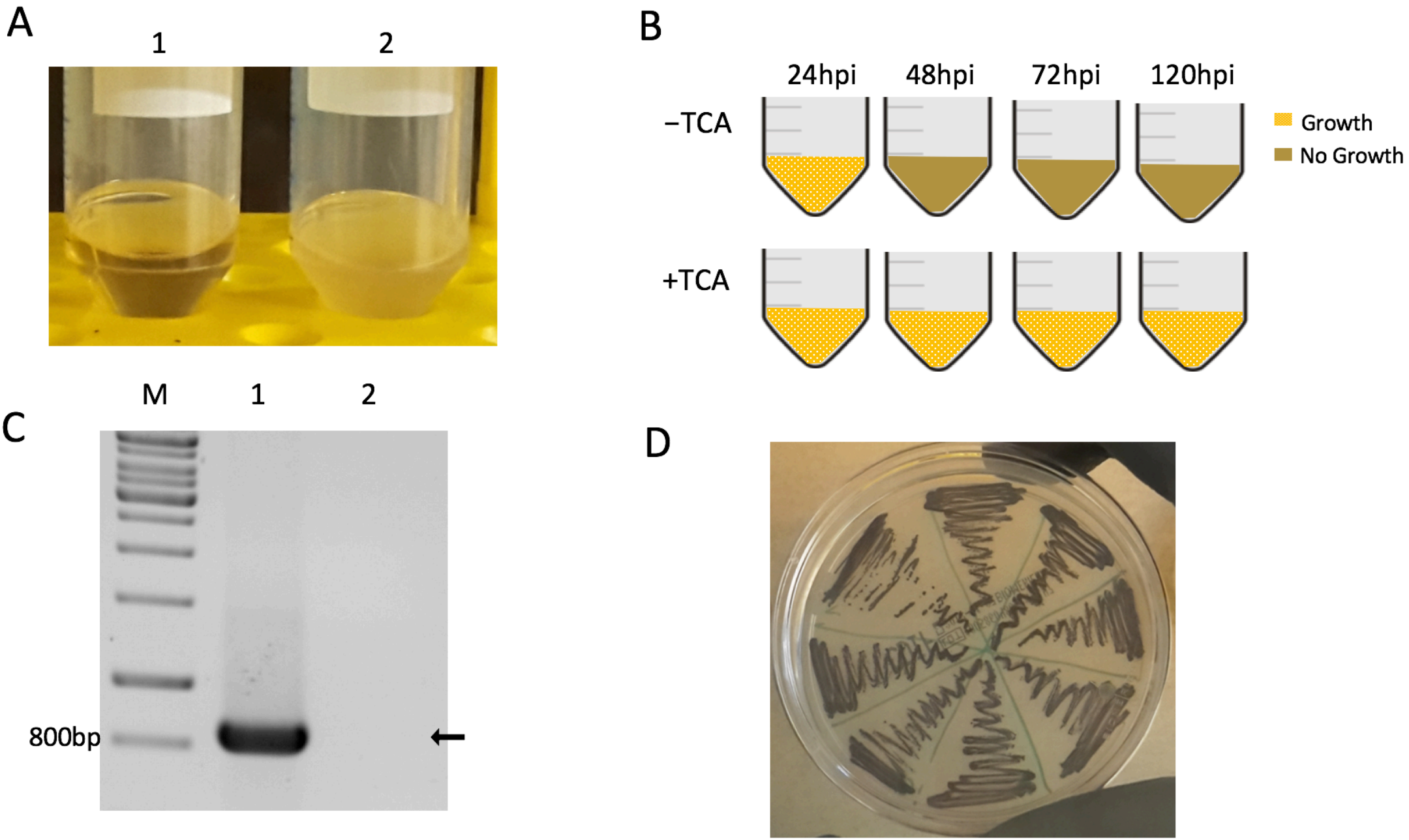
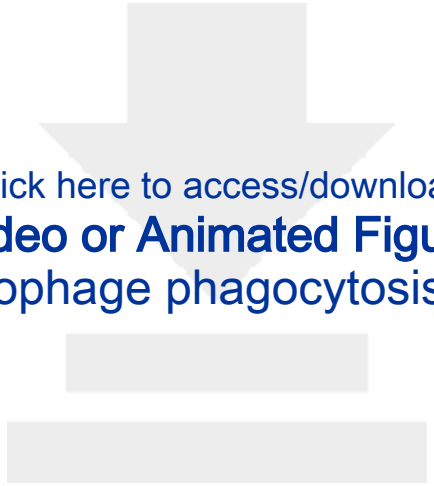


Video or Animated Figure



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Video or Animated Figure

macrophage phagocytosis.mov

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Agarose	Sigma-Aldrich	A2576	Ultra-low gelling agarose
Agarose low-melting (LM)	Pronadisa	8050	It is used in agarose plates
BacLight Red Bacterial Stain	Thermo Fisher Scientific	B35001	Fluorescent dye
Brain-Heart-Infusion Broth	Carl Roth GmbH	X916.1	
Brass (wild-type)			deficient in melanin synthesis, used to generate stable transgenic li
Calcium nitrate ($Ca(NO_3)_2$)	Sigma-Aldrich	C1396	
Capillary Glass	Harvard Apparatus	30-0019	Injection needles
Clostridioides difficile			R20291,, a ribotype 027 strain, TcdA+/TcdB+/CDT+ production
DMSO	Carl Roth GmbH	A994	
FIJI	open-source platform		Image processing
HEPES	Carl Roth GmbH	6763	
Horizontal needle puller	Sutter instrument Inc	P-87	
L-cysteine	Sigma-Aldrich	168149	
Leica Application Suite X (LAS X)	Leica		Image processing
Magnesium sulfate ($MgSO_4$)	Carl Roth GmbH	P026	

Micro injector	eppendorf	5253000017
Microinjection molds	Adaptive Science Tools	TU1
Leica SP8 confocal microscope	Leica	
Phenol Red	Sigma-Aldrich	P0290
Potassium chloride (KCl)	Carl Roth GmbH	5346
Sodium chloride (NaCl)	Carl Roth GmbH	9265
Taurocholate	Carl Roth GmbH	8149
Tg(lyZ: KalTA4)bz17/Tg(4xUAS-E1b:EGFP)hzm3		stable transgenic line in which in which the lyZ promoters drive the
Tg(mpeg1.1: KalTA4) ^{bz16} /Tg(*4xUAS-E1b:EGFP)* ^{hzm3}		stable transgenic line in which in which the mpeg1.1 drive the expre
Tricaine	Sigma-Aldrich	E10521
Yeast extract	BD Bacto	212750

nes

expression of EGFP fluorescent protein in neutrophils

ession of EGFP fluorescent protein in macrophages

We thank all referees for their constructive criticism. We have now substantially revised our manuscript in line with the referee's comments.

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.

We have examined the manuscript with great care to correct the spelling and grammar mistakes.

2. Please revise lines 190-193, 250-252, 283-286, 294-295, 346-348, and 350-351 to avoid textual overlap with previously published work.

We have revised these lines to avoid overlap with previous publications.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Sigma- Aldrich, Sutter Instruments Inc., Harvard Apparatus, Adaptive Science Tools, ThermoFisher, BacLight, etc.

We have deleted all commercial language and brand names as the editor referred, BacLight, Leica SP8, Sigma-Aldrich, Sutter Instruments Inc., Harvard Apparatus, Adaptive Science Tool. The reagents and instruments are listed in the table of materials.

4. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

We have revised line 94 to avoid personal pronouns and line 107, 111, 197 to avoid colloquial phrases.

5. Please revise the Protocol to contain only action items that direct the reader to do something (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." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

We have revised line 105, 184 to use imperative tense.

6. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We have gone through protocol to make sure each step has no more than 3 actions.

7. 1.1: What volume of Danieau medium is used?

We have added that 10ml of Danieau medium is used in line 91.

8. 2.4.1: When are the cultures harvested? What volume of PBS is used to wash?

The cultures were harvested at 16 hours after inoculation. Bacteria were washed with 1 mL PBS.

9. 3.1: Please define dfp. How many larvae are used? How large is the Petri dish?

We have defined dpf as days post-fertilization, and added the number of larvae and the size of the Petri dish in line 143 and line 146.

10. 3.4: Please specify the size of the needle.

We have added the size of the microinjection needle at step 1.2.

11. 4.1: Please use a superscripted number for the reference (Pham et al., 2008).

We have used a superscripted number at line 173.

12. 4.2: Please specify the gnotobioc conditions.

We apologized that a typo caused the misunderstanding and we have corrected it as gnotobiotic conditions.

13. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

We have now highlighted the protocol text that should be covered by the video in yellow.

14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

We carefully went through the highlighted text to make sure a logical flow and at least one action per step is presented.

15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We also highlighted the sub-steps containing the necessary details.

16. Please reference the macrophage phagocytosis movie in the manuscript.

We have added one sentence to refer the movie 1 in line 275.

17. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

We have added the two softwares that were used, Fiji and Leica X, also the *C. difficile* strain, and the zebrafish stains were added.

18. References: Please do not abbreviate journal titles; use full journal name.

We have changed the abbreviated journal titles into their full names.

We thank all reviewers for having read our manuscript with great care, and we are thankful for the helpful advice. Below we will provide answers to all concerns raised by the reviewers in a point by point manner. We hope to clarify the raised questions satisfactorily and point out the individual changes that we incorporated into the manuscript, which we believe has significantly improved with the reviewer's help.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript by Li et al. describes a protocol to inoculate larval zebrafish with C. difficile by injection into the GI tract or oral microgavage, followed by observation of bacterial interactions with phagocytes by fluorescence microscopy, or determination of gut colonization by C. diff culturing from homogenized gut samples or 16S rRNA amplification.
Although protocols describing infection of zebrafish, including with intestinal pathogens have been published, there has been debate about whether the larval gut would be sufficiently low in oxygen to support the growth of strictly anaerobic species. Therefore, a protocol describing infection of zebrafish larvae with C. diff. which would demonstrate the feasibility of colonization with an obligate anaerobe, as well as provide a way to study this clinically relevant pathogen and its interaction with phagocytes in vivo, would be of great interest.

Major Concerns:
The manuscript needs some substantial improvements to unambiguously show that C. diff indeed colonizes the gut, at least in germfree larvae, rather than just transiently passing through the GI tract. Below are a list of suggestions for additional representative results to include that would demonstrate that colonization is indeed the outcome of this protocol (see comment 18).
Additionally, there is a list of suggestions to make the protocol more specific and therefore easier to reproduce, since in its current form some critical details are omitted.

We thank the reviewer for the deailed response and the many helpful advice, which we have attempted to integrate into the manuscript. Regarding the colonization of *C. diff* of the intestine, we share the view of the other reviewers that inoculating the gut directly with actively growing cells and recovering *C. diff* colonies from spores isolated from the GI tract three days later clearly argues against a transient passage of *C. diff* through the gut.

Nevertheless, the purpose of JoVE manuscripts is foremost to present and teach new methodological approaches, and we are convinced that the several reoutes and analytical protocols for working with *C. diff* in zebrafish are new and helpful for the community.

Minor Concerns:
1. Larval (5dpf) zebrafish are used as a model, therefore the title should be expanded to "Development of a larval zebrafish infection model for Clostridioides difficile".

We have changed our title accordingly.

2. Introduction: It should be made clear that C. diff exists in two forms - vegetative spores and actively growing cells, and that infections are done with cells rather than spores.

We have now pointed out that *C. difficile* form spores, and that we have used actively growing *C. difficile* cells, not spores, for this study.

3. Infection in hospital settings often happens by ingestion of spores that persist in the environment. Have the authors tried to gavage larvae with spores to see if they will germinate in the gut and colonize? This should be mentioned in the discussion section.

We indeed tried to infect gnotobiotic larvae with *C. difficile* spores. However, we obtained the same result as for active *C. difficile* cells, in which infected zebrafish did not show symptoms of CDI but *C. difficile* spores were detected.

4. Infection routes described are microinjection into the intestine and microgavage into the mouth. Have water immersion or food-borne infection been tested as alternative routes of infection? If so, what was the outcome? If not, why not? It would be good to include mention of this in the discussion. In particular, another group has achieved C. diff colonization of larval fish by immersion. This work should be referenced (Valenzuela et al., 2018).

We initially tested immersion of zebrafish in *C. difficile* cells, but these tests did not seem to colonize the zebrafish intestinal tract and did not elicit an innate immune response. This is in contrast to the findings reported in Valenzuela et al., 2018, which we now cite in our protocol. The only explanation that we currently have for this discrepancy is that E3 medium used by Valenzuela et al. may favor infection by immersion compared to Danieau medium used in our study. In addition, we did not further follow immersion protocols, as such incubation does not allow to precisely determine the time of infection, which is why we aimed to achieve a better temporal control over pathogen administration.

5. l.89: What is the composition of 30% Danieau medium?

The composition of 30% Danieau is 0.12 mM $MgSO_4$, 0.18 mM Ca $(NO_3)_2$, 0.21 mM KCl, 1.5 mM HEPES pH 7.2, 17.4 mM NaCl. We have now added the receipt in line 91-93.

6. L.116f.: What C. diff. strain was used by the authors? What temperature was used for growth in BHIS liquid medium? How many hours was the broth culture grown for over night?

C. difficile R20291 stain was used, which is now mentioned in line 124. Bacteria were cultured at 37 °C under anaerobic conditions and the culture was grown for 16 h.

7. L.125: What OD600 was typically achieved in the BHIS culture prior to PBS wash and BacLight staining? Was the OD600 adjusted to a specific density prior to BacLight staining, or only after? It seems the initial density prior to staining would affect the intensity of staining.

Typically, ODs of 1-1.2 were achieved by culturing using this method. The OD was adjusted prior to the staining. This is now mentioned in the protocol in line 138 and 139.

8. L.134: What zebrafish line was used for the experiments?

Wild-type, Brass and two stable transgenic lines, *Tg(mpeg1.1: KalTA4)*^{bz16}/Tg(*4xUAS-E1b:EGFP)*^{hzm3} and *Tg(lyZ: KalTA4)*^{bz17}/Tg(*4xUAS-E1b:EGFP)*^{hzm3} were used for the experiments. This is mentioned in lines 281 and 304 respectively.

9. L.134: How as the tricaine solution prepared? Was it buffered and how?

Tricaine powder is dissolved in double-distilled water and adjusted pH to 7 with 1 M Tris-HCL solution, then store at 4 °C. This is now mentioned in line 143-145.

10. Step 3.4. should be highlighted in yellow and included in the video.

We have now highlighted step 3.4. in yellow.

11. L.146. Freshly pulled microinjection needles are usually calibrated with mineral oil prior to use to ensure injection of the correct volume. Is this not done or just not described as part of the protocol?

We have now added more details about how to calibrate the injection needle in step 1.2.

12. Step 3.6. should be highlighted in yellow and should be shown as part of the video, or a corresponding cartoon/animation should be included in the video to demonstrate the location of the injection site in relation to the larval anatomy.

We have now highlighted step 3.6. in yellow to include it in the video presentation.

13. L.190: Step 5.8. should be highlighted in yellow and be included in the video to demonstrate correct microgavage.

We have now highlighted step 5.8. in yellow to include it in the video presentation.

14. L.214: The section about dissection of zebrafish larval intestines should start with a one-sentence introduction to describe that this procedure is used to recover viable bacteria to determine bacterial burden or profile bacterial communities in the zebrafish intestine following C. diff. colonization. Is it necessary to dissect out the intestine, or are similar results obtained with whole-larvae homogenates? Please include this in the discussion.

We revised the title of step 7 as 'Dissection of Zebrafish Larval Intestine to Recover Viable *C. difficile*'. We have also added one introductory sentence in line 232 to emphasize that the intestinal dissection is necessary for the recovery of the viable *C. difficile*. This explanation is repeated in the discussion in lines 371-372.

15. L.249 should read 5dpf.

We have now corrected this sentence.

16. L.304: please give the absolute CFUs microgavaged into the larvae, rather than CFUs/ml.

We have now included the volume of the *C. difficile* that were injected. With a concentration of the inoculum of 10^8cfu/mL (line 139) the absolute CFUs can be approximately determined.

17. L.311: describe in the legend what the 800bp band corresponds to. Details of primers used for PCR amplification should be included in the protocol.

The 800 bp band corresponds to the *C. difficile* specific 16S rDNA band that is produced using the primers:

Cdiff16Sfw: 5' GTG AGC CAG TAC AGG 3'
Cdiff16Srev: 5' TTA AGG AGA TGT CAT TGG 3'
We have added the sequence of primers in step 7.5.

18. It would be useful to include quantitative data of how many CFU of C. diff could typically be recovered from larval guts at various time points post infection.

We agree with this point, but injection of inoculum varies with slight differences in capillary openings, and variability in OD within a certain range. Also slight difference in larval age could affect the outcome, thus at best an approximation could be performed. As the aim of this manuscript is not to report studies about *C. diff* infection dynamics in zebrafish, but to demonstrate the general feasibility of a method and to provide possible technological approaches we think that we have covered the scope of the journal by showing that *C. diff* can be applied to the zebrafish intestine by microgavage and subsequently successfully recovered over several days. These approaches therefore lay the technical ground for a later thorough analysis of infection dynamics.

19. Is the issue of C. diff imaging at later time points really because of bleaching? In our experience, backlight stained bacteria in the gut of zebrafish can be imaged over at least 24 hours without bleaching. The issue may rather be that the bacteria grow and the dye is diluted as a result of cell division. Quantitation of C. diff burden over time (as suggested in 18) will also shed more light on this issue.

We were surprised to learn that Baclight stained bacteria in the gut of zebrafish can be imaged over 24 hours. However, if the dye is not bleached, dilution of the bacteria could be one explanation. Given our results that we could not recover actively growing cells later that 24 hours post-infection, but that spores could be isolated on the following days, we think that spore formation may be responsible for the loss of staining. The sporulation process is divided into several stages, during which physiological land marks change, for example, the assembly of the coat possible affecting labeling intensity. We now mention this possible explanation in the discussion in line 291.

20. L.348: It would be useful to include an image of fixed fish with bacteria stained post-fixation, to unambiguously visualize the presence of both C. diff cells and spores in the gut.

We intended to perform such whole mount immunostaining using an antibody against *C. difficile*. But no appropriate antibody was available for this purpose.

21. L.358: Which are the two anaerobic species the authors are referring to here?

They are Lactobacillus *paracasei and Eubacterium limosum* and we have included them in line 389.

Reviewer #2:

Manuscript Summary:

The authors describe two alternative injection routes to infect the zebrafish intestinal tract with C. difficile, an important anaerobic human pathogen commonly associated with gastrointestinal infections in hospitalised settings. Very few reports have previously adopted microgavage techniques in zebrafish to directly deliver bacteria to the intestine. Additionally, the work described here is particularly challenging because of the bacteria being anaerobic (which also makes it impossible to conventionally trace them with fluorescence reporters which require oxygen to fluoresce). The manuscript is therefore very valuable because it details how to deliver pathogens to the intestine of larval zebrafish and how to track them in vivo (although only for a short period).

Notably, applying this model, the authors suggest sporulation of C. difficile in vivo in the zebrafish. They also have data suggesting active invasion. As the interaction with the microbiome is very important in C. difficile pathogenesis in humans, it is also a very important finding that the natural zebrafish microbiome also can affect the optimal establishment of C. difficile infection in the zebrafish intestine. In turn, the relative ease to rear zebrafish larvae in gnotobiotic conditions until the delivery of the infection (compared to e.g. mice) holds great promise to exploit this model for further research in C. difficile pathogenesis.

Broadly, the injection method described here can be useful to a large spectrum of researchers interested in gastrointestinal pathogens and to other researchers attempting to track anaerobic bacteria in vivo.

We appreciate this positive view about our manuscript and hope to clarify all remaining questions.

I have only minor concerns, mostly related to textual adaptations to clarify protocols and interpretation of the representative results (see comments below).

Major Concerns:

No major concerns to address

Minor Concerns:

- L 62-64 Establishing a transparent zebrafish model (...) "is required" --> The sentence feels like an overstatement. I'd suggest rephrasing.

We have now rephrased the sentence to avoid overstatement. The sentence reads now: ”Therefore, establishing a transparent zebrafish model to reveal the innate immune cell response to *C. difficile* directly inside a living vertebrate organism would facilitate such innate immune cell studies.”

- L 99-100 Can the authors write more precisely how they measure the diameter of the tip of the needle to be 10-20 um?

We now include more details of the measurement of the tip of the needle, which ranges within 30-40 µm. This information can be found in step 5.2.

- L 129 Can the authors also include the volume of PBS in which to wash the C. diff (as this may affect the intensity of final staining)?

The bacteria were washed in 1 mL PBS.

- L 134 It should probably be "0.02-0.04 % Tricaine (Tricaine, Sigma-Aldrich) in 30% Danieau medium" and not Tricaine alone.

Thank you for pointing it out. We have now corrected this point.

- L 143 Where is the 0.5 % phenol red solution from and is it made in PBS?

Yes, in PBS. We have now added it to the protocol.

- L 150 Can the author include how they determine the volume of the drop? Eg. Do they inject in a drop of mineral oil and measure the diameter of the drop using a graticule to then determine the volume?

Yes, we have now included the details about drop size measurement in step 1.2.

- L 153 The fish are embedded in agar. Is the agar scraped off the injection area before injection, or is the needle pierced through the agar too? Doesn't this lead to needle clogging?

The needle pierced through the agar and it didn't cause needle clogging. We have now revised the text to be more precise.

- L 162 Are the fish maintained into gnotobiotic conditions just until the injection procedure and then transferred into standard medium and standard maintenance protocols? If so, it might be useful to state it clearly here.

We have now modified the text to precisely indicate how we maintained the fish and mention under point 4.2:

4.2 All gnotobiotic zebrafish larvae are maintained under gnotobiotic conditions until 5 dpf or before the gavage. After gavage, zebrafish larvae are transferred into a standard incubator, but with sterile 30% Danieau medium.

- L 169 Can the authors include needle diameter here too as they do for the protocol above?

We have now added that the needle diameter is 30-40 µm.

- L 187 As mentioned for the protocol above, can the author include how they measure the volume of inoculum?

We have now added more details in step 1.2.

- L 190 As mentioned for the protocol above, is needle operated also through the layer of agar or is it necessary to remove this from the area?

The needle passed through the agar. We have revised the text in this part.

- L 196 How are the larvae removed from agarose?

The larvae were released from agarose with a flexible microloader tip. We have now added it into the text, which now reads:

5.9 Following gavage, rescue the infected zebrafish larvae from the agarose with a flexible microloader tip by first cutting the agarose away and then by lifting the larvae, transfer these larvae into sterile 30 % Danieau medium.

- L 271 "Therefore, another approach to detect C. difficile was established." I am not sure what this is referred to, where is this other approach described?

We have now revised the sentence as "an intestinal dissection method was established to detect *C. difficile*."

- L 283-289 This sentence is unclear. Can C. difficile be harvested from the intestine >24hpi and can it not be harvested before this time point? If so, it is unclear to me how C. difficile is not detectable before 24h but it is detectable later on. Can the authors write more on how exactly they think that the indigenous microbial communities prevent C. difficile invasion and make it undetectable early upon injection, but not in later stages? Is it because the microbiome delays colonisation (but this can still happen later on) and collection/washing of intestines before 24h results in loss of all the delivered bacteria before they could invade?

We have revised this sentence to be more precise: "However, *C. difficile* was only detected before 24 h post-infection both with and without TCA".

- L 291 It is not clear to me why the bacteria grow at 24h in TCA- medium and then stop growing later on. This should be clarified. Is this because TCA is necessary for spore germination and after 24h all residual C. difficile is only in the form of spores?

This suggestion is what we think happens based on our findings that cells can only be isolated until around 24 hours post-infection, while afterwards only spore-derived bacterial colonies could be obtained. We have added a sentence "which suggested that total *C. difficile* in the gut had formed spores" in line 319-320 to clarify this conclusion.

- L 338-339 Following from the point above, do the authors have evidence that C. difficile proliferates in the intestine of zebrafish? Is it possible that the staining is lost because of proliferation/dilution too? Does the staining used here remain on C. difficile spores?

One of the reviewers made a similar suggestion (Reviewer #1, Minor Concerns 19) and we agree that spore formation including changes in the membrane could be the cause for the loss of fluorescence. Currently we have no indication for pronounced proliferation of *C. diff*.

- Figures: Can the authors include labels for colours and timepoints in all the images? Also, the legend for Figure 1 should inform of the injection route, and weather 1A is bacteria already within the fish or in culture. Figure 3 should state clearly that these fish were gnotobiotic at the time of the gavage. Also, I feel it would have been very valuable to have a more complete figure showing also the result for conventionalised (non-gnotobiotic) zebrafish, as these are discussed in the text (but not shown).

We have now included labels for colors and timepoints in Figure 1 and Figure 2. We have also revised the legend of Figure 1 and 3 to indicate the *C. difficile* used in zebrafish and gnotobiotic zebrafish larvae. Given that we found that conventional zebrafish could not maintain *C. difficile* in the gut for longer than 24h, we did not follow this further, which is why we would not like to show these results here.

- Table of material: it could be handy to have the C. difficile strain information here too.

We have added the information of *C. difficile* strain in Table of material.

Typos:
- Throughout, generally percentages and temperature notations do not require spaces eg. 30 % --> 30%; 4 °C --> 4°C.

We have corrected the space between numbers and percentages. However, the Instruction for Authors says:" Include a space between all numbers and the corresponding unit", so we kept the space for temperature notations.

- Some minor inconsistencies in style for the references throughout. eg. compare L55 and L65 with L53 and L60 (presence/absence of spaces)
- L 120 inconsistent spacing between digits and units throughout, eg. 1 g/L vs 15g/L.
- L 119 "filtratino" --> filtration
- L 196 steril --> sterile
- L 249, L 279 dfp --> dpf

Thank you for pointing out the typos. We have now carefully corrected all of them.

Reviewer #3:
Manuscript Summary:
The authors injected fluorescently labeled C.difficile into 5 dpf zebrafish gut and observed infiltration of neutrophils and macrophages with phagocytosis. To mimic a natural course of infection, the authors also used microgavage to deliver the labeled bacteria in the gut. However the bacteria lose the fluoresence label after 5 hpi and there are minimal immune response. The authors then generated gnotobiotic zebrafish and performed microgavage again. This time, no imaging was performed and bacteria can be recovered from dissected gut up to 120 hpi. However, whether the bacteria can replicate and cause disease, tissue damage or innate activation is not reported. Overall, the work is valuable providing a new way of labeling the bacteria and attempts in establishing the zebrafish infection model.

We are grateful for this positive judgment about our manuscript.

Major Concerns:
The major concern is that the bacteria detection method can not test for CFU or whether the bacteria can establish productive infection after inoculation.
Figure 2 will be more informative if the experiment is performed with the gnotobiotic zebrafish where infection is more likely to be successful.
lack of the evidence that this bacteria can establish productive infection in zebrafish despite various attempts is a major concern.

We agree with the reviewer that a better way to detect *C. difficile* would be helpful to directly follow the behavior of these pathogenic cells and allow for quantitative analysis. We have attempted for quite some time to generate florescent protein expressing *C. difficile*, but we could not obtain convincing fluorescent labeling yet. We will maintain our efforts and hope to be able to provide further conclusive data about *C. difficile* behavior soon.

Minor Concerns:
line 90, receipt of 30% Danieau medium missing. The volume and how the dissolved solution is stored is missing.

We have added the recipe for 30% Danieau and the storing conditions in line 91-93.

a catalog number of normal agarose will be helpful.

We have added the catalog number behind of normal agarose at line 111.

line 119, typo, filtratino

Thank you for pointing out these typos, which we have corrected now.

line 125, how to monitor the initial amount of bacteria before labeling is missing. what is the volume of PBS used for washing?

The bacterial concentration was adjusted using optical density. Bacteria were stained at an OD600 of 1. One mL of PBS was used. We have added this information to the manuscript now.

line 130, how many CFU/ml is equivalent to OD600=1?

OD600 is approximately equivalent to 10^8 cfu/mL. We included this in the manuscript in line 139

illustration or video of dissection of larval intestine will be informative

We have highlighted step 7.3 to demonstrate the dissection during the video recording.

methods of 16S rRNA PCR is missing.

We now provide the following information:

For the 16S-PCR a colony was resuspended in 50 µL of H_2O and boiled at 95 °C for 15 min. Lysed debris as pelleted by centrifugation (14000 rpm, 2 min, RT) and 2 µL of the supernatant were used as template in a 25 µL PCR-reaction using *C. difficile*-specific primers (s.a.). When bacteria from liquid culture were used, 1 mL of culture was harvested, and once washed with 1 mL PBS (14000 rpm, 2 min, RT). The pellet was resuspended in 100 µL of H_2O_{dd} and treated as above.

Reviewer #4:
Manuscript Summary:
In general, this is a well-conducted research that can be consider for publication in JOVE. This protocol aims to established a non-invasive microgavage protocol to deliver C. difficile directly into the intestine of zebrafish larvae by intubation through the open mouth. This novel infection method closely mimics the natural infection route of C. difficile in humans. Therefore, using zebrafish-C difficile ineraction can help in decipher those virulence and host factors important for pathogenesis of this important pathogen. The use of gnotobiotic zebrafish larvae to predispose the zebrafish to be infected is a great idea that can be follow in other enteropathogens infections in this host model.

We are thankful for this positive judgment about our work.

Major Concerns:
My major concern is related with the absent of details regarding the production of gnotobiotic zebrafish larvae. Despite the authors refer this section to a well-established method, in a journal like JOVE all methodological details are of a great importance. I suggest that a summary of the published protocols by Pham et al., 2008 should be included.

We have inserted a summary of the protocol in the Protocol section to highlight the key steps.

This part can now be found in a new chapter 4.

Minor Concerns:
Include more references that compare injections with other immersion methods in zebrafish larvae in other bacterial pathogens is highly desirable in the discussion section. For example BMC Immunol. doi: 10.1186/1471-2172-12-58; Front Cell Infect Microbiol. doi: 10.3389/fcimb.2017.00334

We have extended the discussion on this point and added these literatures as references.