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## Video Article

# Deciphering and Imaging Pathogenesis and Cording of *Mycobacterium abscessus* in Zebrafish Embryos

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## Abstract

Zebrafish (*Danio rerio*) embryos are increasingly used as an infection model to study the function of the vertebrate innate immune system in host-pathogen interactions. The ease of obtaining large numbers of embryos, their accessibility due to external development, their optical transparency as well as the availability of a wide panoply of genetic/immunological tools and transgenic reporter line collections, contribute to the versatility of this model. In this respect, the present manuscript describes the use of zebrafish as an *in vivo* model system to investigate the chronology of *Mycobacterium abscessus* infection. This human pathogen can exist either as smooth (S) or rough (R) variants, depending on cell wall composition, and their respective virulence can be imaged and compared in zebrafish embryos and larvae. Micro-injection of either S or R fluorescent variants directly in the blood circulation *via* the caudal vein, leads to chronic or acute/lethal infections, respectively. This biological system allows high resolution visualization and analysis of the role of mycobacterial cording in promoting abscess formation. In addition, the use of fluorescent bacteria along with transgenic zebrafish lines harbouring fluorescent macrophages produces a unique opportunity for multi-color imaging of the host-pathogen interactions. This article describes detailed protocols for the preparation of homogenous *M. abscessus* inoculum and for intravenous injection of zebrafish embryos for subsequent fluorescence imaging of the interaction with macrophages. These techniques open the avenue to future investigations involving mutants defective in cord formation and are dedicated to understand how this impacts on *M. abscessus* pathogenicity in a whole vertebrate.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/53130/>

## Introduction

*Mycobacterium abscessus* is an emerging pathogen that causes a wide spectrum of clinical syndromes in humans. These include cutaneous infections as well as severe chronic pulmonary infections, mostly encountered in immunocompromised and in cystic fibrosis patients<sup>1,2,3,4</sup>. *M. abscessus* is also regarded as a major rapidly-growing mycobacterial species responsible for nosocomial and iatrogenic infections in humans. Moreover, several recent reports highlighted the possibility that *M. abscessus* could cross the blood-brain barrier and induce important lesions in the central nervous system (CNS)<sup>5,6</sup>. Despite being a rapid grower, *M. abscessus* exhibits also several pathogenic features that are related to those of *Mycobacterium tuberculosis*, including the capacity to remain silent for years within granulomatous structures and to generate caseous lesions in the lungs<sup>7</sup>. More alarming is the low sensitivity of *M. abscessus* to antibiotics, rendering these infections extremely difficult to treat leading to a significant therapeutic failure rate<sup>8,9</sup>. The important threat of this species is mainly its intrinsic resistance to antibiotics, which is of major concern in public health institutions<sup>10</sup> and a contraindication to lung transplantation<sup>11</sup>.

*M. abscessus* displays smooth (S) or rough (R) colony morphotypes that lead to different clinical outcomes. In contrast to the S strain, R bacteria have a tendency to grow end to end, leading to a rope or cord-like structure<sup>12,13</sup>. Several independent studies based on either cellular or animal models revealed the hyper-virulence phenotype of the R morphotype<sup>14,15</sup>. From epidemiological studies, the most severe cases of *M. abscessus* pulmonary infections appear to be associated with R variants<sup>16</sup> which are the only variant that has been seen to persist for years in an infected host<sup>3</sup>. The morphotype difference relies on the presence (in S) or loss (in R) of surface-associated glycopeptidolipids (GPL)<sup>12</sup>. However, due to the inherent limitations of the currently available cellular/animal models used to study *M. abscessus* infection, our knowledge regarding the pathophysiological events of the R or S variants remains obscure. Infection of immuno-competent mice *via* intravenous or aerosol routes leads to transient colonization, impeding the use of mice to study persistent infections and for *in vivo* drug susceptibility testing<sup>17</sup>. Therefore, developing animal models amenable to the manipulation of the host response is a major challenge. In this context, non-mammalian models of infection have been developed recently, including *Drosophila melanogaster*<sup>18</sup> that offers several advantages such as cost, speed and ethical acceptability over the mouse model. The zebrafish (*Danio rerio*) model of infection has also been explored to visualize, by non-invasive imaging, the progression

and chronology of *M. abscessus* infection in a live animal<sup>19</sup>. Importantly, a proof of concept was also established to demonstrate its suitability for *in vivo* antibiotic assessments against *M. abscessus*<sup>17,20</sup>.

The zebrafish have been widely used during the last two decades to study the interactions between various pathogens and the host immune system<sup>21</sup>. The increasing success of this alternative vertebrate model relies on major and unique opportunities that motivated and validated its use for a better understanding of numerous viral and bacterial infections<sup>19,22,23,24,25,26,27,28,29</sup>. As opposed to most other animal models, zebrafish embryos are optically transparent, allowing non-invasive fluorescence imaging<sup>30</sup>. This has led to study *M. abscessus* infected zebrafish embryos with unprecedented details, culminating with the description of extracellular cording, that represent an example of bacterial morphological plasticity. Cording represents a new mechanism of subversion of the immune system and a key mechanism promoting pathogenesis of acute *M. abscessus* infection<sup>19</sup>.

This report describes new tools and methods using the zebrafish embryo to decipher the pathophysiological traits of *M. abscessus* infection and to study the intimate interactions between the bacilli and the innate immune system. First, a detailed microinjection protocol that includes processing of the bacterial inoculum, embryo preparation, and infection *per se*, is presented. Methods specifically adapted to assess *M. abscessus* virulence by measuring various parameters, such as host survival and bacterial burden, are presented. Special focus is given on how to monitor, at a spatiotemporal level, the fate and progression of the infection and the host immune response to *M. abscessus* using video microscopy. Moreover, to investigate the contribution and role of macrophages during *M. abscessus* infection, methods to generate macrophages-depleted embryos (using either genetically- or chemically-based approaches) are described. Finally, protocols to visualize the specific interactions with macrophages or neutrophils using either fixed or living embryos are documented.

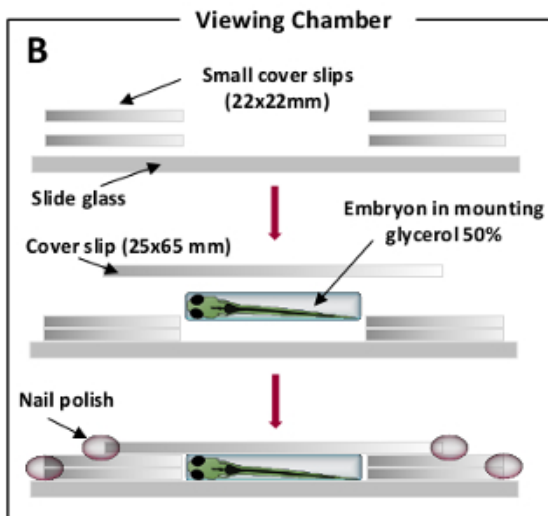
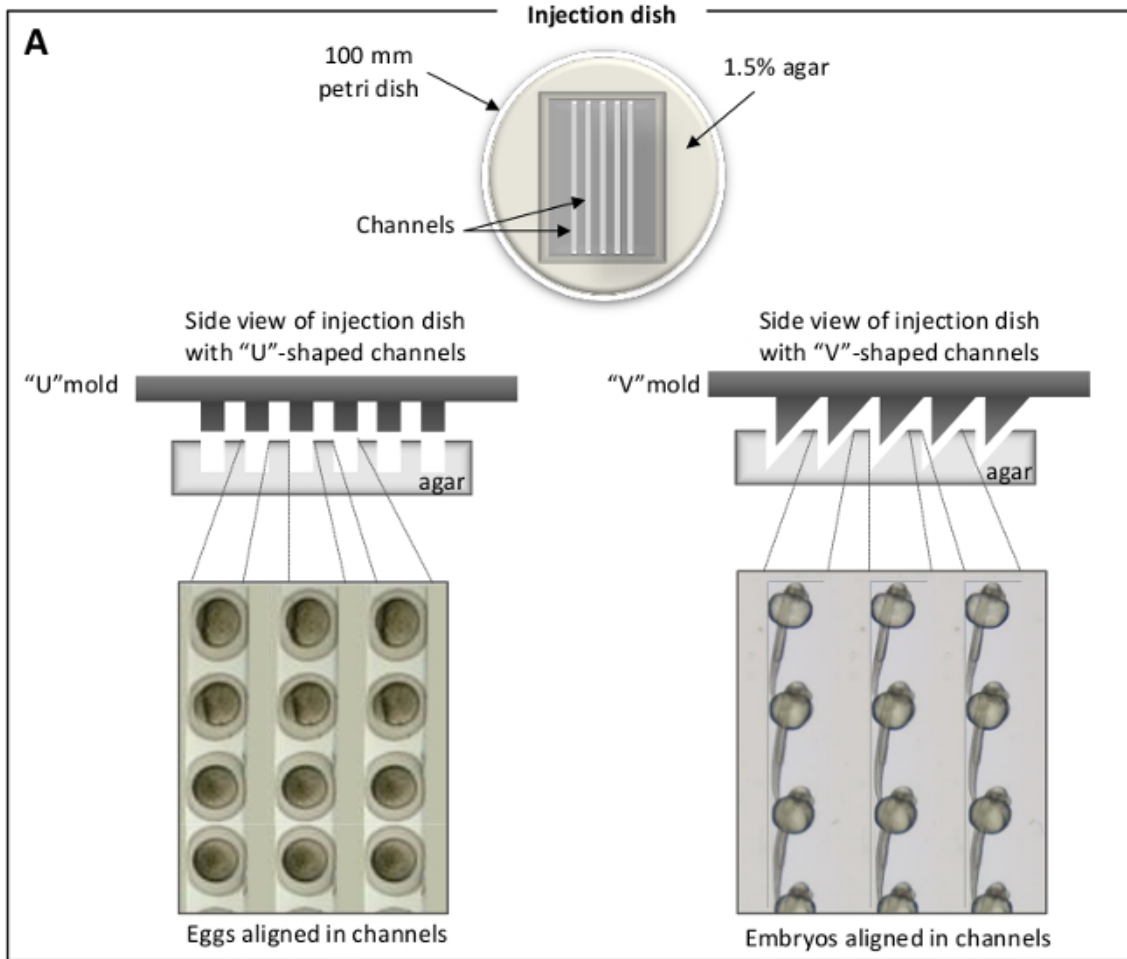
The aim of this report is to stimulate further studies to shed new light into *M. abscessus* virulence mechanisms and particularly the role of cording in the establishment of an acute and uncontrolled infection process.

## Protocol

Zebrafish experimental procedures must comply with the relevant institutional and governmental regulations. For the present study, zebrafish experiments were done at the University Montpellier, according to European Union guidelines for handling of laboratory animals ([http://ec.europa.eu/environment/chemicals/lab\\_animals/home\\_en.htm](http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm)) and approved under the reference CEEA-LR-13007.

### 1. Preparation of Reagents and Microinjection Equipment

1. Prepare fish water by dissolving 0.06 g Instant Ocean Sea salt in 1 L distilled water<sup>31</sup>, then autoclave to sterilize (120 °C for 20 min) and store at 28.5 °C for up to 1 month.
2. Prepare methylene blue solution by dissolving 1 g of methylene blue in 1 L distilled water. Add 300 µl of methylene blue solution in 1 L fish water to obtain blue fish water, then autoclave to sterilize and store at 28.5 °C for up to 1 month.  
NOTE: The addition of methylene blue in fish water prevents mold growth.
3. Preparation of Phosphate Buffered Saline (PBS)
  1. Prepare a 10 X PBS stock solution by dissolving 5.696 g Na<sub>2</sub>HPO<sub>4</sub>, 680 mg KH<sub>2</sub>PO<sub>4</sub>, 969 mg KCl and 78.894 g NaCl in 1 L distilled water and adjust the pH to 7.0 with HCl. To obtain 1 X PBS, dilute 100 ml of the 10 X PBS solution in 900 ml distilled water and autoclave during 20 min at 120 °C to sterilize.
  2. Add 0.05% Tween 80 to obtain 1 X PBST and store at RT for up to 1 year.
4. Prepare oleic Acid Dextrose Catalase (OADC) enrichment by dissolving 8.5 g NaCl, 50 g Bovine Serum Albumin, 20 g D-Glucose, 40 mg Catalase from bovine liver and 0.5 g of oleic acid in 1 L distilled water then filter sterilize the solution and store it at 4 °C for up to 6 months.
5. Prepare 100 ml of ethyl 3-aminobenzoate methanesulfonic acid (tricaine) stock solution, by dissolving 400 mg tricaine powder in 97.9 ml distilled water. Add 2.1 ml of 1 M Tris (pH 9) to adjust the pH at 7.0 and store the solution at -20 °C.
6. Prepare the microcapillary injection needles by pulling borosilicate glass capillaries (1mm O.D. X 0.78 mm I.D.) using a micropipette puller device with the following settings : Air pressure 650; Heat 999; Pull 50; Velocity 80; Time 200.
7. Prepare a 1.5% agar solution in blue fish water and pour 25 ml of melted agar in 100 mm petri dishes. On the top of the melted agar, place homemade molds in order to imprint specific channels. "V"-shaped channels are used to position embryos while the "U"-shaped are used for eggs (**Figure 1**)<sup>31</sup>. Once solidified, remove carefully the imprint.  
NOTE: Cover the agar with blue fish water to avoid dehydration and store at 4 °C for up to 2 months.

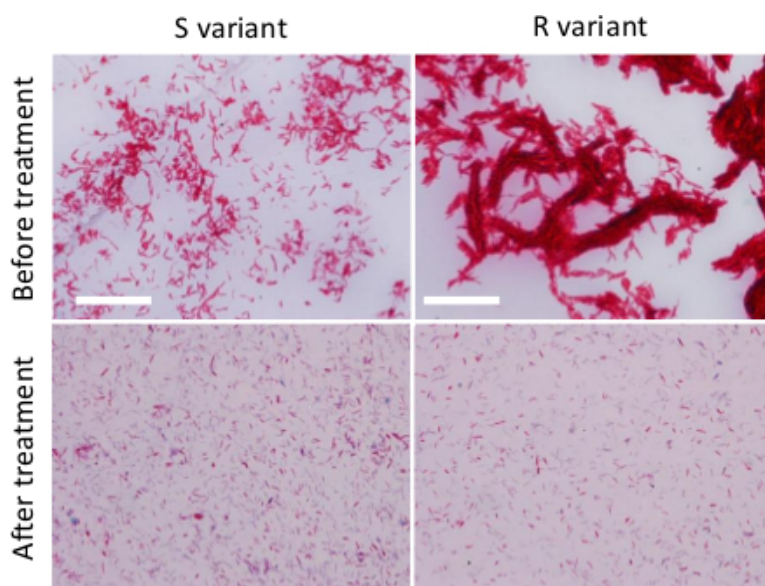


**Figure 1. Positioning chambers for zebrafish injections.** (A) U-shaped channels for eggs (left panel) and V-shaped channels for embryos (right panel). Zebrafish eggs/embryos are laid in the channels and aligned along the same axis. (B) Viewing chambers for microscopic observation. [Please click here to view a larger version of this figure.](#)

## 2. Preparation and Storage of the *M. abscessus* Inoculum

1. *M. abscessus* growth conditions
  1. Plate out *M. abscessus* from a -80°C glycerol stock onto a Middlebrook 7H10 agar containing 10% of OADC and 0.5% glycerol (7H10<sup>OADC</sup>) and supplemented with the appropriate antibiotic depending on the resistance marker carried by the vector encoding the fluorescent protein. Incubate the plates at 30 °C for 4-5 days.

2. Pick up a fluorescence-positive mycobacterial colony and inoculate 1 ml Middlebrook 7H9 broth supplemented with OADC, 0.2% glycerol, 0.05% Tween 80 (7H9<sup>OADC/T</sup>) with the required antibiotic in a 15 ml sterile plastic tube. Incubate at 30 °C for 1 week without shaking.  
NOTE: Tween 80 restricts clumping and bacterial aggregation.
  3. Resuspend the pre-culture obtained in 2.1.2 in 50 ml Middlebrook 7H9<sup>OADC/T</sup> in 150 cm<sup>2</sup> tissue culture flasks to a final 0.1 OD<sub>600</sub> (corresponding to around 5.10<sup>7</sup> bacteria/ml) and incubate further at 30 °C for 4 days to obtain an exponentially-growing culture (OD<sub>600</sub> = 0.6 to 0.8).  
NOTE: To minimize clumping, especially of the rough variant, incubation should not exceed 5 days. Both smooth and rough variants display a similar growth rate.
2. Preparation of the *M. abscessus* inocula  
NOTE: Due to high propensity of rough *M. abscessus* to form large aggregates and to produce cords, a specific treatment is necessary to generate homogeneous and quantitatively controlled inocula prior to microinjections in the embryos. This treatment is also applied to smooth bacteria that produce aggregates, albeit to a lesser extent than the rough strain.
    1. Harvest exponentially-growing cultures from 150 cm<sup>2</sup> tissue culture flasks by centrifugation at 4,000 x g for 15 min at RT in a 50 ml sterile plastic tube and resuspend the bacterial pellet in 1 ml 7H9<sup>OADC/T</sup>. Aliquot 200 µl of bacterial suspensions into 1.5 ml microcentrifuge tubes.  
NOTE: Working with small volumes in 1 ml syringes is necessary to obtain highly homogeneous suspensions.
    2. Homogenize the bacterial suspensions with a 26-G needle (15 up-and-down sequences), sonicate three times for 10 sec (with 10 sec breaks between each sonication sequence) using a waterbath sonicator. Add 1 ml of 7H9<sup>OADC/T</sup> and vortex briefly. Centrifuge 3 min at 100 x g.
    3. Carefully collect the mycobacteria-containing supernatants to avoid the clumps and pool the homogenous suspensions in a 50 ml sterile plastic tube.
    4. Check visually for the presence of eventual remaining bacterial aggregates.  
NOTE: If aggregates are still present, proceed to an additional centrifugation step at 100 x g for 2 min, and collect the supernatant.
    5. Centrifuge the suspension at 4,000 x g for 5 min, resuspend the pellet in 200 µl 7H9<sup>OADC/T</sup> and proceed to the injection.
    6. Assess the final bacterial concentration by plating serial dilutions (in 1 x PBST) on 7H10<sup>OADC</sup> agar and counting colony forming units (CFU) after 4 days incubation at 30 °C. CFUs should be determined for each new batch.
    7. Prepare frozen inocula stocks by storing 5 µl aliquots at -80 °C.  
NOTE: The CFU assessment of the -80°C frozen aliquots is a pre-requisite to determine the exact number of viable bacteria as freeze/thawing may affect bacterial viability of the inoculum. These frozen inocula are ready to be used for subsequent injections. Since all aliquots contain the same number of CFU, they allow injecting bacteria in a reproducible manner from one experiment to another.
  3. Inoculum quality control  
NOTE: Ziehl-Neelsen staining (specific to mycobacteria) can be used to compare the quality of the bacterial suspensions prior to and after the homogenization procedures described in 2.2.
    1. Spot and spread out one droplet of the homogenized bacterial suspension on a glass slide and allow it to dry completely, fix the smear for at least 30 min on a hot plate set to 65-70 °C and stain using a Ziehl-Neelsen staining protocol.
    2. Observe the bacterial smear with a microscope using a 100 X objective and compare with a smear of a non-processed bacterial suspension (**Figure 2**).



**Figure 2. Preparation of dispersed *M. abscessus* inocula.** Ziehl-Neelsen staining of R or S variants grown on broth medium prior to any treatment (upper panels) or after treatment (lower panels) to reduce the size and number of mycobacterial aggregates (successive steps of syringing, sonication and decantation). Bacteria were observed using a microscope with 100 X APO oil 1.4 NA objective). Scale bars: 20 µm. [Please click here to view a larger version of this figure.](#)

### 3. Preparing Zebrafish Embryos

1. Spawning and collecting zebrafish eggs
  1. The day before spawning, breed adult zebrafish by placing 2 males and 1 female (usually a 2:1 ratio allows optimal fertilization rate) into a breeding chamber, consisting of a separate fish tank with an egg collection basket<sup>31</sup>.  
NOTE: Egg collection baskets are used to protect the eggs from being eaten by the parents and facilitate egg harvesting.
  2. At 1 hpf, harvest eggs and only properly developed embryos in a 100 mm Petri dish filled with 25 ml blue fish water (maximum 100 embryos per dish) and keep them at 28.5 °C. Non-fertilized eggs are discarded.
2. Preparation of zebrafish embryos for injections
  1. At about 24 hr post-fertilization (hpf), dechorionate the embryos with fine tweezers in a 100 mm Petri dish and keep them at 28.5 °C.
  2. Collect the 30-48 hpf embryos using a pipette and transfer them to a "V"-shaped positioning chamber filled with 25 ml of fish water containing 270 mg/L tricaine. Lay the embryos properly in the channels using a homemade microloader tip tool (homemade clipped micro loader tip) optimized for an easier micro-manipulation.  
NOTE: Orientate the dorsal side downwards for intravenous injections in the caudal vein or the dorsal side upwards for tail muscle injections.

### 4. Micro-injection Procedure

NOTE: The micro-injection procedure for *M. abscessus* is similar to the one described previously for *M. marinum* injections<sup>32</sup>. To assess the chronology of *M. abscessus* infection (survival, bacterial loads, kinetic and characteristics of infection), injections in the caudal vein of 30 hpf embryos are preferred. To visualize the recruitment of immune cell, injections are done in localized sites such as is in tail muscles in 48 hpf embryo.

1. Dilute the mycobacterial inoculum in 1 X PBST (depending on the number of CFU to be administered as determined in Step 2.2.6), containing 10% red phenol to check proper injection.
2. Load a microcapillary injection needle with 5-10 µl of the bacterial inoculum using a microloader tip, connect the microinjection needle into the holder of the three-dimensional micromanipulator connected to the injector and break off the top of the needle with fine tweezers to obtain an opening diameter of 5-10 µm.
3. Calibrate the injection volume by adjusting the microinjection pressure (20-50 hPa) and time (0.2 sec).  
NOTE: The required injection volume is calibrated by visual determination of the diameter of a droplet expelled into the yolk of an embryo.
4. For caudal vein injection, position the embryo with the ventral side facing the needle (as described in section 3.2.2) and place the needle tip close to the urogenital opening, targeting the caudal vein, and gently push the tip of needle into the embryo until it just pierces the caudal vein region. Deliver the desired volume of bacterial preparation, usually 1-3 nl containing around 100 CFU/nl.
5. For intramuscular injection, position the embryo with the dorsal side facing the needle (as in section 3.2.2), place the needle over one somite and inject a small volume (1 nl) of inoculum.  
NOTE: As for caudal vein injections, intramuscular infections are also challenging to perform. Care must be taken to avoid an overloading that may injure the surrounding tissues.
6. At the end of the injection procedure, control the size of the inoculum by injecting the same volume of bacterial suspension in a drop of sterile PBST followed by plating on 7H10<sup>OADC</sup> and counting the CFU. Around 100 embryos can be injected with a needle.  
NOTE: Because rough *M. abscessus* may continue to form aggregates in the needle, injections need to be done immediately after preparation of the inoculum.
7. Transfer the infected fish individually in 24-well plates containing 2 ml/well fish water and incubate at 28.5 °C. Embryos injected with 1-3 nl 1 X PBST can be used as controls.
8. Proper infection of fluorescent bacteria in embryos is monitored using a fluorescence microscope.

### 5. Generation of Macrophage-Depleted Embryos

NOTE: Selective depletion of macrophages from tissues is used to investigate their contribution and role during infection. To visualize the proper depletion of macrophages, it is recommended to use a transgenic line with fluorescent macrophages, where mCherry is specifically expressed under the control of the macrophage specific *mpeg1* promoter<sup>19</sup>.

1. Lipo-clodronate-based procedure  
NOTE: The lipo-clodronate procedure allows a selective depletion of macrophages from tissues (but no neutrophils)<sup>19</sup>. This compound does neither alter the survival of embryos nor affect the integrity of bacteria. A detailed protocol to prepare liposome-encapsulated clodronate has been reported previously<sup>33</sup>.
  1. At 24 hpf, dechorionate the embryos and transfer them to a "V"-shaped injection dish filled with fish water supplemented with tricaine to maintain the embryos in an anesthetized state until the end of the injection procedure, as described in 3.2.2. Orientate the dorsal side downwards.
  2. Load a microcapillary injection needle with liposome-encapsulated clodronate (lipo-clodronate), connect the microinjection needle into the holder of the three-dimensional micromanipulator and break off the top of the needle with fine tweezers to obtain a tip opening diameter of 10 µm.
  3. To calibrate the injection volume, adjust the microinjection pressure to 20 hPa and the injection time to 0.2 sec. Place the needle tip close to the urogenital opening, targeting the caudal vein, and gently push the tip of needle into the embryo until it just pierces the caudal vein region and deliver the desired volume of solution (2-3 nl, 3 times).

NOTE: The lipo-clodronate suspension is very sticky and should be vortexed prior to injection to avoid blocking the vascular system and killing of the embryo. It is crucial to proceed to several successive injections of small volumes.

4. Transfer the embryos in 100 mm petri dishes containing fish water and keep them at 28.5 °C until infection.
5. Control the proper depletion of macrophages by fluorescent microscopy.

NOTE: Complete macrophage depletion is effective for 4 days after lipo-clodronate administration.

## 2. Morpholino-based procedure

1. The day before spawning, breed adult zebrafish by placing 2 males and 1 female separated by a plastic barrier in a breeding chamber.
2. The next day, ≈ 30 min after that the light turns on in the zebrafish facility, remove the barrier separating males and females. Wait for about 20 min after beginning of the spawning to optimize the fertilization rate. Harvest the eggs and slow their development by transferring them in a 100 mm petri dish filled with cold blue fish water (4 °C).

NOTE: The control of the spawning is crucial for the morpholinos-based experiments. Morpholinos can only be injected into the eggs up to the four cells stage.

3. Collect the eggs using a pipette and deposit the eggs down in the “U”-shaped injection-chamber filled with cold blue fish water and block the eggs in the channels using a homemade microloader tip tool.
4. Prepare the *pu.1* morpholino solution containing 10% of red phenol, as previously described<sup>19</sup>. Load a microcapillary injection needle with the morpholino preparation and connect the microinjection needle into the holder of the three-dimensional micromanipulator, then break off the tip of the needle with fine tweezers to obtain a tip opening diameter of 5-10 μm.
5. To calibrate the injection volume, adjust the microinjection pressure to 20-50 hPa and the injection time to 0.2 sec. Place the needle tip close to the egg and gently push the tip of needle through the chorion into the egg and deliver the desired morpholino solution volume, typically 3-5 nl.
6. After micro-injection, transfer the eggs in a 100 mm petri dish in fish water and incubate at 28.5 °C till the infection procedure begins.
7. Analyze the proper (complete) depletion of macrophages in *pu.1* morphants by fluorescent microscopy at 30 to 48 hpf.

NOTE: Depending on the concentration injected, *pu.1* morpholinos may also affect the number of early neutrophils. To confirm the proper depletion of macrophages without altering the number of neutrophils, it is recommended to use a double transgenic zebrafish line with fluorescent macrophages and neutrophils (with GFP expression specifically driven by the *mpx* promoter)<sup>19</sup>.

## 6. Bacterial Burden Quantification

### 1. Determination by CFU enumeration

1. At the desired time point, collect groups of infected embryo (5 per conditions) in 1.5 ml microcentrifuge tubes (1 embryo/tube), cryo-anesthetize the embryos by incubation on ice for 10 min and euthanize using an overdose of tricaine (300-500 mg/L)
2. Wash the embryos with sterile water twice and dispense them in a new tube, lyse each embryo with 2% Triton X-100 diluted in 1 X PBST using a 26-G needle and homogenize the suspension until complete lysis. Centrifuge the suspension and resuspend the pellet in 1 X PBST with 0.05% Tween 80. Serial dilutions of the homogenates are plated on Middlebrook 7H10<sup>OADC</sup> and supplemented with BBL MGIT PANTA, as recommended by the supplier.

NOTE: *M. abscessus* being more susceptible to NaOH treatment than *M. marinum*, decontamination of fish lysates without affecting *M. abscessus* growth can be successfully achieved using the BBL MGIT PANTA antibiotic cocktail.

3. Incubate the plates for 4 days at 30 °C and count colonies.

### 2. Determination by Fluorescence Pixel Count (FPC) measurements in live embryos

NOTE: To determine the bacterial loads *via* fluorescence emission, serial images of infected embryos are acquired and fluorescence intensity measured by FPC (identification of bacteria by particle analysis) using a homemade macro developed for the ImageJ freeware. A particle analysis requires a binary black and white image that relies on a threshold range that allows to discriminating the fluorescent signal of interest from the background.

1. At the desired time point, tricain-anesthetize infected embryos (5-10 per condition) in 35 mm petri dishes as described in 3.2.2.
2. Remove water and submerge the embryos and the whole dish surface with 1% low melting point agarose in fish water, then align laterally the embryos. Cover the solidified agarose with fish water containing tricaine.
3. Acquire fluorescence images of the entire embryo using an epifluorescence microscope with a 10 X objective.  
NOTE: The fluorescence image acquisition is a critical step of the FPC measurements procedure. It is important to use a calibrated monitor with a colour calibration probe. Adjusting the optimal time of exposure to obtain a minimal background during acquisition prevents saturation of the signal. Images must be in an 8-bit tiff format. Identical settings are kept during the whole experiment for quantitative purposes.
4. Remove carefully the agarose from the embryos with a homemade microloader tip tool. These embryos can be used for subsequent analyses if required. Prior to the quantification of the bacterial load, each picture shall be quality controlled. To analyze the images and convert the fluorescence intensity into FPC per fish, open the ImageJ freeware.  
NOTE: The FPC value reflects the bacterial burden as shown previously using *M. marinum*<sup>34</sup>.
5. Determine the required threshold for image analysis using an image of an uninfected embryo (several control embryos can be used to obtain an average threshold). Go to Image → Adjust → Threshold and then move the bottom slider in the threshold window to the right until the background becomes completely black (*i.e.*, to obtain a background should be at zero). Record the corresponding value.
6. Within Image J, open the FPC macro (supplementary file) and follow the prompts: locate the folder of images to be measured and then enter the threshold as previously determined and click on “OK”.

NOTE: The macro automatically subtracts the background. A threshold is then applied. Florescent signals are identified following the particle analysis.

7. Copy and paste the data from the summary window to the desired software for data analysis.

NOTE: In contrast to the CFU determination method, the FPC method is non-invasive and allows re-use the embryos for subsequent analyses and, importantly, monitoring the dynamics/kinetics of the bacterial burden on an individual basis. It, therefore, allows to significantly reducing the number of animals, in agreement with ethical rules.

## 7. Imaging *M. abscessus*-infected Embryos

1. Live imaging of *M. abscessus* infection
  1. Mount tricaine-anesthetized embryos (see 3.2.2) in 1% low melting point agarose in a 35 mm petri dish prior to epifluorescence microscopy observations. Use a glass bottom dish for inverted confocal microscopy or in a single cavity depression slide for upright confocal microscopy. Orientate the embryo to the desired position and cover the solidified agarose with fish water containing tricaine.
  2. Use an epifluorescence microscope with a 10 X objective for sequential fluorescence acquisition and transmission images of the entire embryo. Alternatively, use a confocal microscope with 40X or 63X objectives to visualize the activity of immune cells after an infection.
2. Imaging fixed *M. abscessus* infected embryos
  1. Euthanize the embryos as described in 6.1.1 and transfer them to 1.5 ml micro-centrifuge tubes and fix the embryos in 4% paraformaldehyde in 1 X PBST for 2 hr at RT or O/N at 4 °C. Remove paraformaldehyde by washing the embryos twice with 1 X PBST for 10 min.
 

NOTE: To preserve fluorescence, fixed embryos must be protected from light.
  2. To preserve the integrity of the tissues and fluorescence, embryos are incubated successively in increasing glycerol concentration solutions (10, 20, 30, 40 and 50% diluted in 1 X PBST), for 10 min per condition.
 

NOTE: Fixed embryos can be stored for several months in 50% glycerol at 4 °C.
  3. Lay the embryo embedded in 50% glycerol in a viewing chamber (**Figure 1B**)<sup>31</sup>.
  4. Image using 40X or 63X objectives on a confocal microscope.

### Representative Results

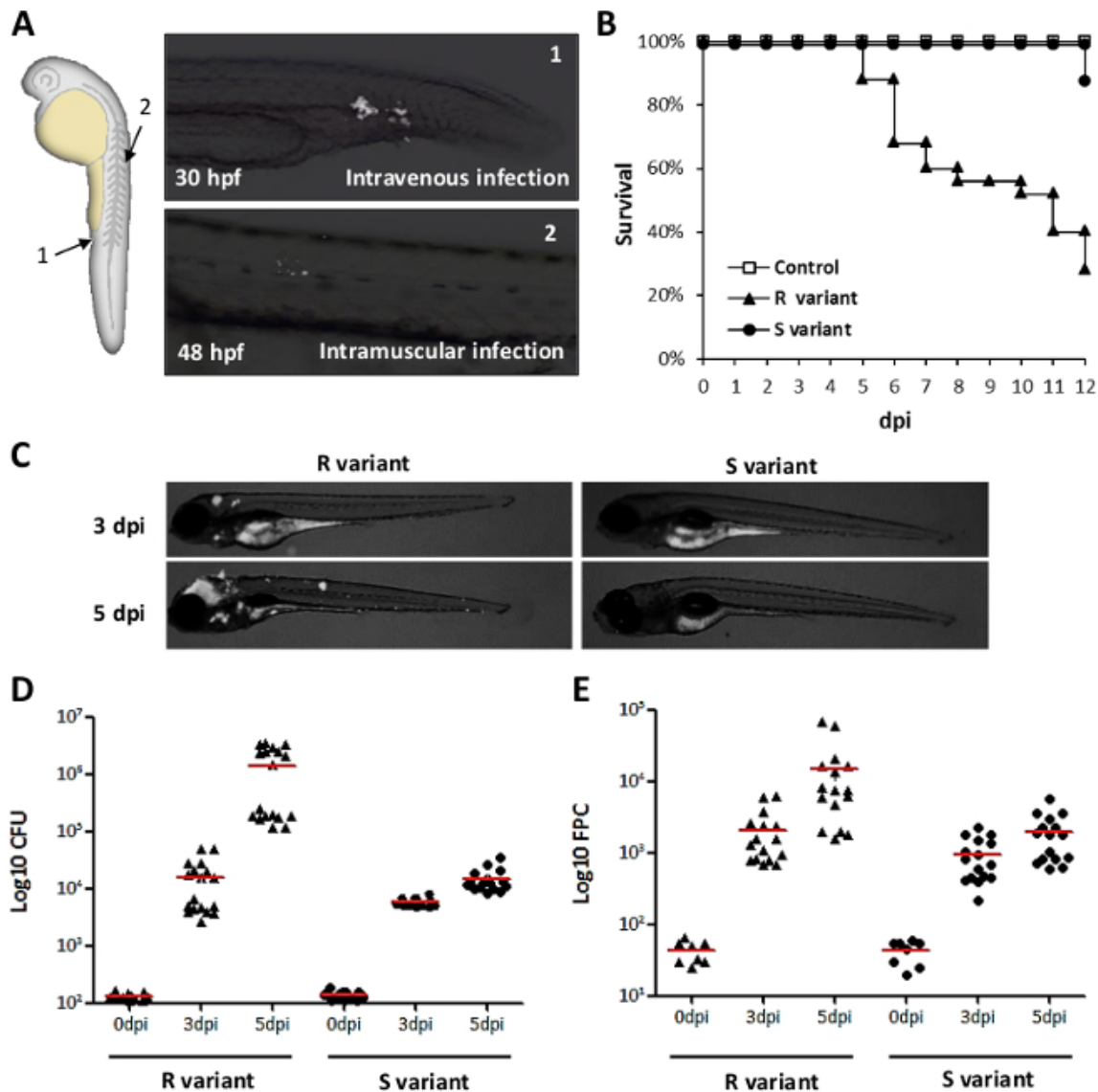
Although various anatomical sites can be injected<sup>32</sup>, caudal vein injections are often used to generate systemic infection for subsequent analyses including survival experiments, bacterial burden determination, phagocytosis activity or cord formation. Injections in the tail muscles are used to assess the recruitment of macrophages at the site of injection (**Figure 3A**). To investigate and compare the virulence of R and S variants of *M. abscessus*, fluorescent bacterial suspensions are injected in the caudal vein of 30 hpf embryos (**Figure 3A**)<sup>19</sup>. In contrast to the S variant, the R morphotype induces a more robust and lethal infection in zebrafish embryos (**Figure 3B**), characterized by the rapid development of bacterial abscesses within the Central Nervous System (CNS) (**Figure 3C**). Systemic injections with both the R and S variants lead to CNS infections and difference in the pathology and virulence phenotypes can be quantified either by fluorescence microscopy observation (**Figure 3C**), by enumerating the CFU per fish (**Figure 3D**) or by determining the FPC of acquired images (**Figure 3E**). Importantly, CFU and FPC determination are correlated and point out to larger bacterial loads for the R strain than the S strain at 3 and 5 dpi. Overall, these results strongly support zebrafish as a relevant and non-invasive model of infection to study the chronology of the infection process.

Perhaps one of the most spectacular features was revealed by video-microscopy that allows visualizing serpentine cords within the CNS of embryos infected with the R variant (**Figure 4**). Thanks to the optical transparency of the embryos, the kinetic of R-cord formation could be monitored and imaged in a non-invasive manner, illustrating the high propensity of the R variant to replicate extracellularly. This uncontrolled replication rate, combined with cellular/tissue destruction rapidly leads to the development of abscesses and ultimately larval death.

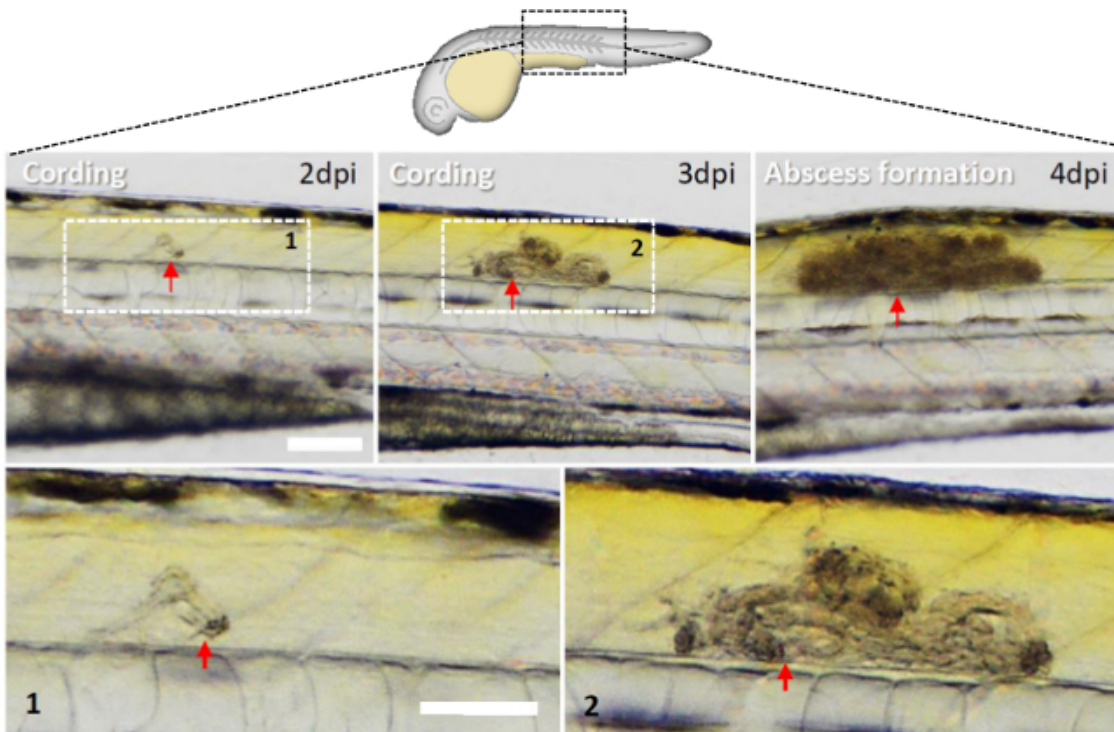
Macrophages are known to play an important role during mycobacterial infection and particularly by driving the granuloma formation<sup>35</sup>. Two complementary strategies can be used to study *M. abscessus* growth/virulence in macrophage-depleted embryos: the lipo-clodronate- and morpholino-based procedures (**Figure 5A**). Infection of macrophage-depleted embryos with the R variant leads to a massive increase in the bacterial loads and cord production as revealed by video-microscopy (**Figure 5B**), leading to extremely rapid death (100% dead embryos at 3 dpi; **Figure 5C**). This clearly indicates that macrophages are required to control *M. abscessus* infection.

To further investigate the role of macrophages during infection, specific transgenic embryos harboring red fluorescent macrophages (*mpeg1:mCherry*) can be used<sup>19</sup>. Injection of *M. abscessus* expressing Wasabi into the tail muscle or in the caudal vein induces a marked recruitment of myeloid cells, mainly macrophages, at the site of infection. This recruitment leads to efficient phagocytosis of individual bacteria (**Figure 6A-6B**). Despite the presence of macrophages, confocal microscopy reveals the appearance of large bacterial cords that macrophages cannot phagocytize (**Figure 6C**). Taken together, these findings highlight a new mechanism of immune evasion based on the role of cording in preventing mycobacterial phagocytosis.

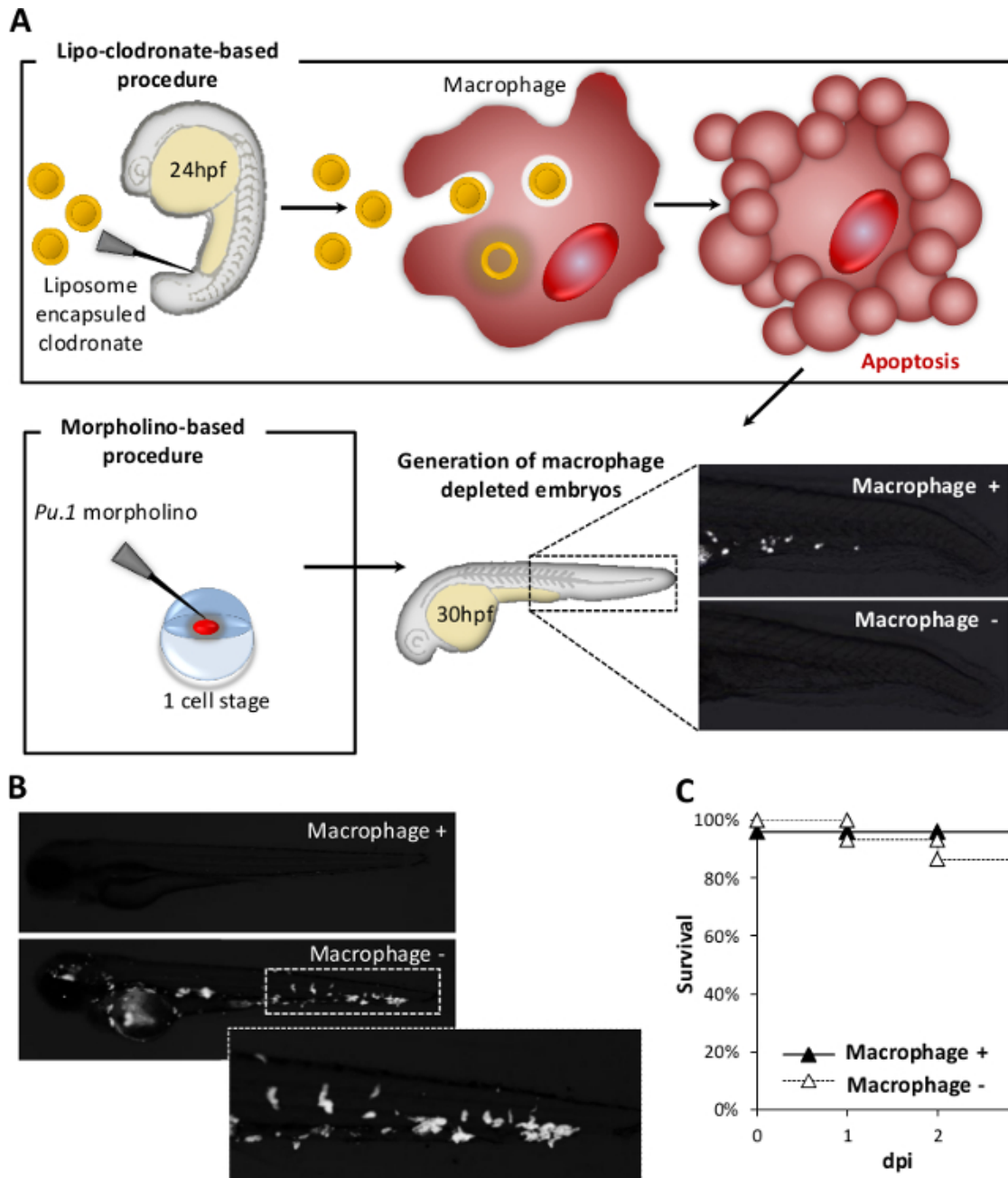




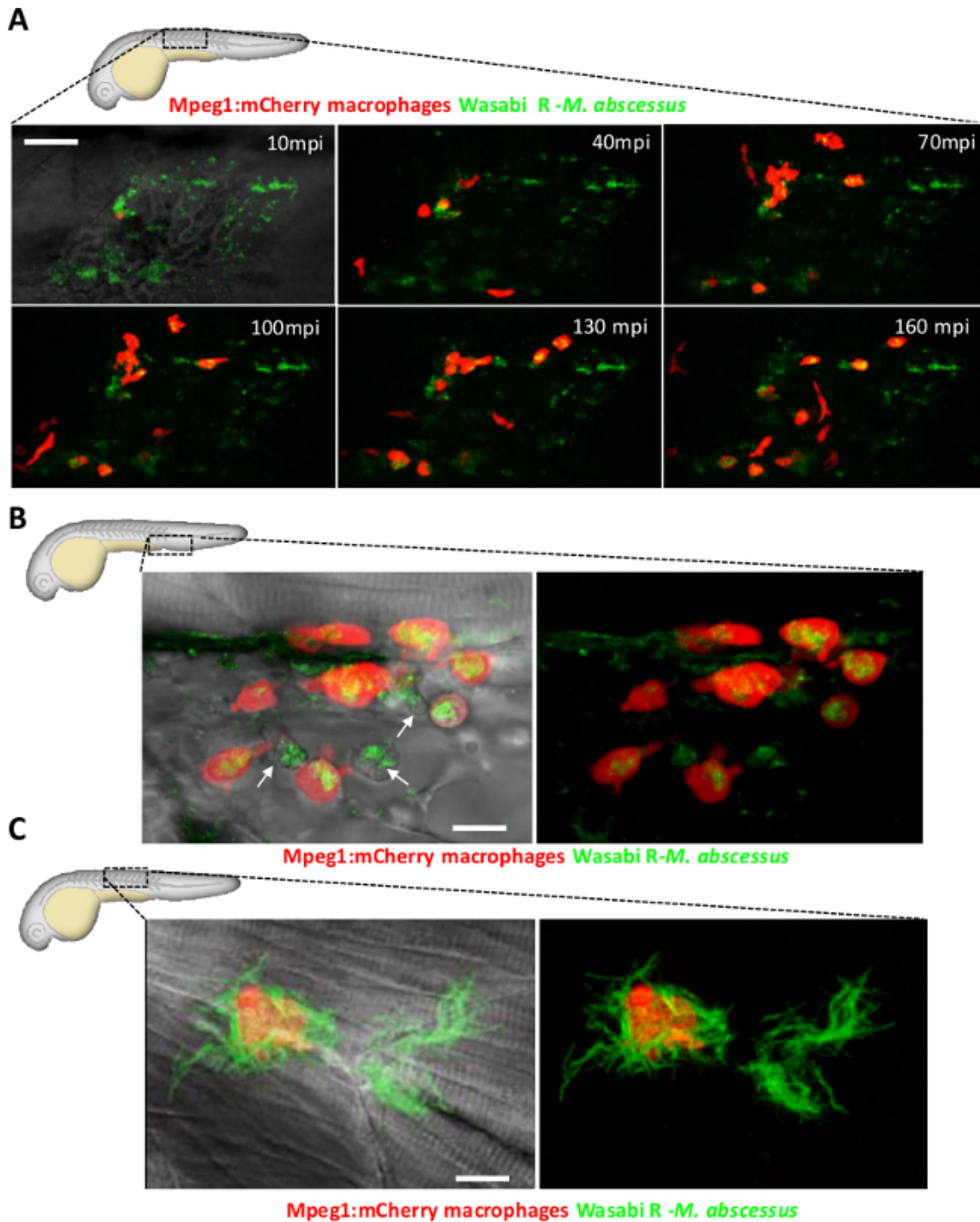
**Figure 3.** *M. abscessus* infection in zebrafish embryos. **(A)** (Top panel) Intravenous infection performed by injecting fluorescent *M. abscessus* (shown in white) in the caudal vein (arrow 1), posterior to the urogenital opening, in 30 hpf embryos. (Lower panel) Intramuscular infection performed by injecting fluorescent mycobacteria (shown in white) in the tail muscle over a somite (arrow 2), in 48 hpf embryos. **(B-E)** 30 hpf embryos intravenously infected with tdTomato-*M. abscessus* (R and S variants). **(B)** Survival curves of embryos infected with  $\approx 300$  CFU either R or S variants or PBS injected controls (n=20). Representative results from three independent experiments are shown. **(C)** Spatiotemporal visualization of either the R or the S variant expressing tdTomato (white) at various times points post-infection performed by fluorescence live imaging. For each strain, a representative fluorescence and transmission overlay of whole infected embryos is shown. The same embryos were imaged at 3 and 5 dpi. **(D-E)** Bacterial loads within embryos infected with either R or S variants ( $\approx 200$  CFU) determined by CFU counting **(D)** or FPC measurements **(E)**. **(D)** CFU from lysate of individual embryos at various time points post-infection determined by plating onto Middlebrook 7H10<sup>OADC</sup> supplemented with BBL MGIT PANTA. Results are expressed as mean log  $\pm$  SD (horizontal bars) CFU per embryo from three independent experiments (n=5). **(E)** FPC measurements from individual living embryos at various time points post-infection based on particle analysis using ImageJ. Results are expressed as mean log  $\pm$  SD (horizontal bars) FPC per embryo from three independent experiments (n=5). All images were obtained with an epifluorescence microscope equipped with a digital color camera. [Please click here to view a larger version of this figure.](#)



**Figure 4.** *In vivo* cording of the R variant. 30 hpf embryos infected intravenously with the R variant of *M. abscessus* and imaged at various time points post-infection to visualize the progression of cord formation. The red arrow is used as a fixed reference point in order to follow the spatiotemporal evolution of the cord. The DIC pictures of the part of the tail containing the growing serpentine cord are boxed (microscope equipped with a color camera). Scale bars: 100  $\mu$ m. [Please click here to view a larger version of this figure.](#)



**Figure 5. Depletion of macrophages leads to increased cord proliferation. (A)** Embryos are depleted using the lipo-clodronate-based procedure or the morpholino-based procedure. (Top panel) Clodronate-encapsulated liposomes (lipo-clodronate) are injected intravenously in 24 hpf *Tg(mpeg1:mCherry)* embryos. Within the circulation, lipo-clodronate is rapidly engulfed by macrophages that undergo subsequent apoptosis. (Left-lower panel) Injection of the morpholino preparation to knock-down the expression of the *pu.1* gene into fertilized *Tg(mpeg1:mCherry)* zebrafish eggs. (Right-down panel) The correct depletion of macrophages in embryos is checked by fluorescence microscopy. **(B-C)** 30 hpf macrophage-containing (+) or macrophage-depleted (-) embryos (following lipo-clodronate treatment) are infected with *M. abscessus* R expressing tdTomato (shown in white). **(B)** Live imaging at 2 dpi shows exacerbation of the infection within macrophage-depleted embryos and hyper-cording. **(C)** Survival curves of the macrophage-containing or macrophage-depleted embryos infected with  $\approx 300$  CFU *M. abscessus* R (n=20). Representative results from three independent experiments are shown. All images were acquired with a microscope connected to a color camera. [Please click here to view a larger version of this figure.](#)



**Figure 6. Behavior of macrophages in embryos infected either locally or systemically with *M. abscessus*.** (A) 48 hpf *Tg(mpeg1:mCherry)* zebrafish embryos are infected intramuscularly with Wasabi-expressing *M. abscessus* R. Live imaging using a confocal microscope to visualize recruited macrophages phagocytizing individual mycobacteria (in minutes post-infection, mpi). Maximum intensity projection showing an intense recruitment of macrophages (red) to the injection site (confocal microscope with 40X APO water 0.8 NA objective). Scale bar: 20  $\mu$ m. (B-C) 48 hpf *Tg(mpeg1:mCherry)* embryos intravenously infected with *M. abscessus* R expressing Wasabi were fixed and imaged by confocal microscopy (40X) at 4 hpi (B) and 3 dpi (C). (B) Maximum intensity projection of macrophage (red) or other myeloid cells, presumably neutrophils (arrows), containing isolated mycobacteria (green) close to the site of injection (confocal microscope with 63X APO oil 1.33 NA objective). Scale bar: 10  $\mu$ m. (C) Maximum intensity projection showing one macrophage (red) unable to phagocytize a cord (green) (confocal microscope with 63X APO oil 1.33 NA objective). Scale bar: 5  $\mu$ m. [Please click here to view a larger version of this figure.](#)

## Discussion

The zebrafish has recently emerged as an excellent vertebrate model system for studying the dynamics of bacterial infection using wide field and confocal imaging in real-time<sup>36</sup>. The combination of dispersed mycobacterial suspensions (protocol 2.2) together with micro-injection methods

(protocol 4) allows reproducible systemic infections, and subsequent monitoring and visualization of the progression of the infection with a special focus on the bacterial interactions with host macrophages. Virulence of *M. abscessus in vivo* can be investigated thanks to the use of wild-type golden zebrafish<sup>37</sup>. To study interactions between *M. abscessus* and host myeloid cells, several transgenic zebrafish lines can be used such as *Tg(mpeg1:mCherry)* to visualize macrophages<sup>19</sup>. Additional transgenic reporter lines such as the *as Tg(mpx:GFP)*<sup>38</sup> or *Tg(lyz:DsRed)*<sup>39</sup> with either green or red neutrophils, respectively, can also be used to address the specific role of these granulocytes in response to the infection.

*M. abscessus*, and particularly the R variant, exhibits a natural propensity to form massive bacterial aggregates<sup>19</sup>, thus preventing reproducible micro-injection. In addition, the fact that the R variant forms much larger clumps than the S variant also precludes the potential comparison of the virulence phenotypes for the two isogenic variants. The protocol described in section 2 allows obtaining homogenous and dispersed *M. abscessus* suspensions for micro-injection in embryos. This preparation can be applied to any other mycobacterial species or mutant strains displaying excessive aggregating properties. Since the sonication step can alter the outermost mycobacterial cell wall layer, care should be taken to assess the impact of this physical treatment on bacterial viability for each new strain.

Determination of bacterial burdens routinely relies on CFU counting after plating lysed embryos on selective agar medium at various time points post-infection, as described in protocol 6.1. This procedure includes several limitations: since animals are euthanized, each embryo (or group of embryos) can be used for the determination of a single time point. In addition, this method remains inaccurate due to a large proportion of bacteria that are killed and/or lost during lysis, subsequent serial dilutions, or mainly by clumping. Counting the CFU is also a laborious task. As an alternative, the protocol 6.2 describes an efficient method to quantify the *M. abscessus* burden that includes limited handling steps, based on the analysis of fluorescent images of infected embryos with pixel quantification, as developed originally for *M. marinum*<sup>40,41</sup>. This FPC method based on particle analysis, shows a good correlation with the CFU countings. Similarly, by combining microscopy and fluorescence quantification, relative changes in innate immune cell numbers per embryo can be quantified by image analysis using adequate transgenic lines.

Zebrafish embryos can be used to evaluate the role of immune cell during infection and macrophage depletion was found to adversely affect host survival in response to *M. abscessus* infection. Specific macrophage depletion can be efficiently generated using the lipo-clodronate-based procedure (protocol 5.1) or the morpholino-based procedure (protocol 5.2). Nevertheless, since the presence of macrophages is crucial for the establishment and the maintenance of the vascular system and for correct embryonic development, it is recommended to ablate macrophage production at 24 hpf using lipo-clodronate.

The zebrafish embryo appears as a unique model to study *M. abscessus* infections and the role of extracellular cording as an immune system subversion strategy. If determinants controlling cording in *M. abscessus* can be identified, they could lead to the development of innovative therapeutic options. Linking the use of zebrafish together with innovative high throughput technologies<sup>42</sup> also opens the field to drug discovery and new pharmacological approaches against this drug-resistant pathogen.

## Disclosures

The authors have nothing to disclose.

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