

Video Article

A Zebrafish Embryo Model for In Vivo Visualization and Intravital Analysis of Biomaterial-associated *Staphylococcus aureus* Infection

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Abstract

Biomaterial-associated infection (BAI) is a major cause of the failure of biomaterials/medical devices. *Staphylococcus aureus* is one of the major pathogens in BAI. Current experimental BAI mammalian animal models such as mouse models are costly and time-consuming, and therefore not suitable for high throughput analysis. Thus, novel animal models as complementary systems for investigating BAI *in vivo* are desired. In the present study, we aimed to develop a zebrafish embryo model for *in vivo* visualization and intravital analysis of bacterial infection in the presence of biomaterials based on fluorescence microscopy. In addition, the provoked macrophage response was studied. To this end, we used fluorescent protein-expressing *S. aureus* and transgenic zebrafish embryos expressing fluorescent proteins in their macrophages and developed a procedure to inject bacteria alone or together with microspheres into the muscle tissue of embryos. To monitor bacterial infection progression in live embryos over time, we devised a simple but reliable method of microscopic scoring of fluorescent bacteria. The results from microscopic scoring showed that all embryos with more than 20 colony-forming units (CFU) of bacteria yielded a positive fluorescent signal of bacteria. To study the potential effects of biomaterials on infection, we determined the CFU numbers of *S. aureus* with and without 10 µm polystyrene microspheres (PS₁₀) as model biomaterials in the embryos. Moreover, we used the ObjectJ project file "Zebrafish-Immunotest" operating in ImageJ to quantify the fluorescence intensity of *S. aureus* infection with and without PS₁₀ over time. Results from both methods showed higher numbers of *S. aureus* in infected embryos with microspheres than in embryos without microspheres, indicating an increased infection susceptibility in the presence of the biomaterial. Thus, the present study shows the potential of the zebrafish embryo model to study BAI with the methods developed here.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58523/>

Introduction

A variety of medical devices (referred to as "biomaterials") are increasingly used in modern medicine to restore or replace human body parts¹. However, the implantation of biomaterials predisposes a patient to infection, called a biomaterial-associated infection (BAI), which is a major complication of implants in surgery. *Staphylococcus aureus* and *Staphylococcus epidermidis* are two most prevalent bacterial species responsible for BAI^{2,3,4,5,6}. Implanted biomaterials form a surface susceptible to bacterial biofilm formation. Moreover, local immune response may be deranged by the implanted biomaterials, causing reduced effectiveness of bacterial clearance. The initial clearance of infecting bacteria is performed mainly by infiltrating neutrophils, which have strongly reduced bactericidal capacity in the presence of an inserted or implanted biomaterial⁷. Moreover, macrophages infiltrating the tissue after the initial influx of neutrophils will phagocytose the remaining bacteria but cannot effectively kill them intracellularly, due to deranged immune signaling that is a consequence of the combined presence of the biomaterial and bacteria⁸. Thus, the presence of biomaterials can facilitate intracellular survival of bacteria^{9,10,11,12,13} and biofilm formation on the implanted biomaterials^{4,14}. Consequently, BAI may lead to the failure and need for replacement of implanted biomaterials, causing increased morbidity and mortality and prolonged hospitalization with additional costs^{2,15}.

An increasing number of anti-BAI strategies are being developed^{2,16,17}. *In vivo* evaluation of the efficacy of these strategies in relevant animal models is essential. However, traditional experimental BAI animal models (e.g., mouse models) are usually costly, time-consuming, and therefore not suitable for high throughput testing of multiple strategies¹⁸. Recent development of bio-optical imaging techniques based on bioluminescent/fluorescent labeling of host cells and bacteria may allow for the continuous monitoring of BAI progression and host-pathogen/host-material interactions in single small animals such as mice^{18,19,20,21}. However, this technique is relatively complex and still in its infancy, and several issues must be addressed for quantitative analysis of BAI¹⁸. For instance, a high challenge dose is required to visualize bacterial colonization. In addition, light scattering and adsorption of bioluminescence/fluorescence signals in tissues of mammalian test animals must also

be addressed^{18,19,21}. Therefore, novel, cost-effective animal models allowing for intravital visualization and quantitative analysis over time are valuable complementary systems for studying BAI *in vivo*.

Zebrafish (embryos) have been used as a versatile *in vivo* tool for dissecting host-pathogen interactions and infection pathogenesis of several bacterial species such as mycobacteria²², *Pseudomonas aeruginosa*²³, *Escherichia coli*²⁴, *Enterococcus faecalis*²⁵, and *staphylococci*^{26,27}. Zebrafish embryos have many advantages such as optical transparency, a relatively low maintenance cost, and possession of an immune system highly similar to that in mammals^{28,29}. This makes zebrafish embryos a highly economic, living model organism for intravital visualization and analysis of infection progression and associated host responses^{28,29}. To allow visualization of cell behavior *in vivo*, transgenic zebrafish lines with different types of immune cells (e.g., macrophages and neutrophils) and even with fluorescently tagged subcellular structures have been developed^{28,29}. In addition, the high reproduction rate of zebrafish provides the possibility of developing high throughput test systems featuring automated robotic injection, automated fluorescence quantification, and RNA sequence analysis^{27,30}.

In the present study, we aimed to develop a zebrafish embryo model for biomaterial-associated infection using fluorescence imaging techniques. To this end, we developed a procedure to inject bacteria (*S. aureus*) in the presence of biomaterial microspheres into the muscle tissue of zebrafish embryos. We used *S. aureus* RN4220 expressing mCherry fluorescent protein (*S. aureus*-mCherry), which was constructed as described elsewhere for another *S. aureus* strain^{10,31}. The transgenic zebrafish line (mpeg1: UAS/Kaede) expressing Kaede green fluorescent protein in the macrophages³² and blue fluorescent polystyrene microspheres were used. In a previous study, we have shown that intramuscular injection of microspheres into zebrafish embryos to mimic biomaterial implantation is feasible³³. To quantitatively analyze the progression of BAI and associated cell infiltration in single embryos over time, we used the "Zebrafish-Immunotest" project file which is operated within "ObjectJ" (a plug-in for ImageJ) to quantify the fluorescence intensity of bacteria residing and macrophages infiltrating in the vicinity of the injection site of microspheres³³. In addition, we determined the numbers of colony-forming units (CFU) of bacteria in the presence and absence of microspheres in the embryos to study potential effects of biomaterials on infection. Our present study demonstrates that with the methods developed here, the zebrafish embryo is a promising, novel vertebrate animal model for studying biomaterial-associated infections *in vivo*.

Protocol

In this protocol, maintenance of adult zebrafish is in compliance with the local animal welfare regulations as approved by the local animal welfare committee. Experiments with embryos were performed according to the 2010/63/EU Directive.

1. Preparation of "Bacteria-only" and Bacteria-microspheres Suspensions

NOTE: The *S. aureus* RN4220 strain expressing mCherry fluorescent protein (*S. aureus*-mCherry) is used. The *S. aureus* RN4220 strain is mutated in the virulence regulator gene *agrA* (accessory gene regulator A)³⁴, and therefore may have relatively low virulence in the zebrafish embryo model. Other *S. aureus* strains or other bacterial species for BAI can be used.

1. **Take 4 to 5 colonies of *S. aureus* RN4220 bacteria from tryptic soya agar culture plates supplemented with 10 µg/mL chloramphenicol and culture the bacteria to mid-logarithmic growth phase in 10 mL of tryptic soy broth supplemented with 10 µg/mL chloramphenicol at 37 °C under shaking.**
 1. During culture, dilute 100 µL of the bacterial suspension in 900 µL of sterile phosphate buffered saline (PBS) in a cuvette (width of 1 cm) for an optical density (OD) measurement at 620 nm (OD₆₂₀). Culture the bacteria until the OD₆₂₀ reaches 0.4–0.8.
NOTE: An OD₆₂₀ of 0.1 generally corresponds to 3.0 × 10⁷ CFU/mL *S. aureus*. The OD₆₂₀ of an inoculum of bacteria in mid logarithmic growth phase is between 0.4–0.8. Different time periods for culturing may be needed for other species and strains of bacteria.
2. Centrifuge bacteria at 3,500 × *g* for 10 min and re-suspend the pelleted bacteria in 1 mL of sterile PBS. Subsequently wash the bacteria with sterile PBS 2 times, and finally re-suspend the bacteria in 1.1 mL of 4% (w/v) polyvinylpyrrolidone₄₀ (PVP₄₀) solution in PBS.
3. Vortex this bacterial suspension and dilute 100 µL of the suspension in 900 µL of sterile PBS in a cuvette for the OD₆₂₀ measurement. Adjust the concentration of the bacterial suspension with PVP₄₀ solution. This is the "Bacteria-only" suspension.
4. Biomaterials can be freely chosen to mix with bacterial suspension. In the present study, commercial polystyrene (PS) microspheres (blue fluorescent, 10 µm) are used. To generate a Bacteria-Microspheres suspension, centrifuge the microspheres for 1 min at 1,000 × *g*, room temperature, discard the supernatant and re-suspend the microspheres in the "Bacteria-only" suspension (in PVP₄₀ solution).
5. In order to inject approximately equal doses of bacteria in the presence and in the absence of microspheres, the concentration of Bacteria-Microspheres suspension needs to be two thirds of the concentration of the "Bacteria-only" suspension (without microspheres). This is achieved by diluting the Bacteria-Microspheres suspension with 0.5 volume of PVP₄₀ solution. Mix the suspensions by vortexing. Of note, this ratio (two thirds) has been assessed for *S. aureus*. It is also appropriate for *S. epidermidis*, but it is advised to check whether this ratio also applies to other bacterial species and other sizes (than 10 µm) or shapes of biomaterials (than microspheres) to be injected.
6. Check the concentration of the "Bacteria-only" and Bacteria-Microspheres suspension by quantitative culture of 10-fold serial dilutions as below: transfer 100 µL of the suspensions to a 96 well-plate and serially dilute by transferring 10 µL aliquots of the suspension into 90 µL of sterile PBS. Plate duplicate 10 µL aliquots of the undiluted and diluted suspensions on agarose plates, incubate the plates at 37 °C overnight, count the colonies and calculate the numbers of bacteria (colony-forming units. CFU).

2. Breeding, Harvesting, and Maintenance of Zebrafish Embryos

1. Follow the general procedures described earlier^{35,36} for breeding, harvesting, and maintenance of zebrafish embryos, with modifications described below. Cross a family of wild type Tupfel long fin (TL) zebrafish or zebrafish of the selected transgenic line (here, Mpeg1: Kaede) in a tank with a net added to induce the adult females to produce eggs after the light turns on, then separate adults from the produced eggs.
2. Collect the embryos the next day and discard the non-transparent ones which are not viable. Keep approximately 60 embryos per Petri dish (100 mm in diameter) in E3 medium³⁷ and incubate at 28 °C. Remove dead and abnormal embryos, and refresh the E3 medium daily.

3. Preparation of Injection Needles

1. Prepare the glass microcapillary needles for injection using a micropipette puller instrument. Use the following settings: heat: 772, pull: 100, vel: 200, time: 40, gas: 75.
2. Break the needle tip with forceps at the position where the needle has an outer diameter of approximately 20 μm (for 10 μm microspheres), using a light microscope with a scale bar in the ocular. Avoid needles with a very large opening size, as they will compromise survival of the embryos.

NOTE: The opening may be chosen to be smaller or larger, depending on the size and shape of biomaterials to be injected. In the literature, injections using needles with an opening of approximately 50 μm have been reported to cause a significant decrease in embryo survival³⁸.

4. Injection of "Bacteria-Only" or Bacteria-microspheres Suspension into Zebrafish Embryos

1. Heat agarose solution (1–1.5% (wt) in demi-water) using a microwave oven and pour into a 100-mm Petri dish. Place a plastic mold template on top of the agarose solution in the Petri dish to create indentations in the agarose for placing embryos in proper positions, facilitating injections. Incubate at room temperature and remove the mold when the agarose solution has solidified.
2. At 3 d post-fertilization, place the embryos in a 100 mm Petri dish containing 0.02% (w/v) 3-aminobenzoic acid (Tricaine) to anaesthetize them. After 5 min, transfer the embryos to the agarose plate overlaid with E3 medium containing 0.02% (w/v) Tricaine and align them in one orientation for injection. For the Mpeg1: Kaede transgenic line first anaesthetize embryos in E3 medium containing 0.02% (w/v) tricaine. Then select embryos expressing green fluorescent proteins using a stereo fluorescence microscope.

3. **Load the needle with approximately 10 μL of the "Bacteria-only" or Bacteria-Microspheres suspension using a microloader pipette tip. Mount the needle onto a micromanipulator connected to the micro-injector. For the injector used here (see Table of Materials), use the following settings for injections of 2–3 nL: pressure: 300–350, back pressure: 0, time: 2 ms.**

NOTE: The settings for the micro-injector depend on the injector used. Injector settings may need to be adjusted for injections of bacteria mixed with biomaterials with other shapes or sizes.

1. Use needles with the same opening for the injection of the "Bacteria-only" suspension and the Bacteria-Microspheres suspension. If the needle is broken or clogged, always change for a new needle for further injections.
4. Insert the needle into the muscle tissue of embryos under a light microscope (**Figure 1**), at an angle of 45–60° between the needle and the body of embryos. Adjust the position of the needle in the tissue by gently moving it back and forth. Inject the embryos using a foot pedal connected to the micro-injector.
5. After injection of fluorescent bacteria, score the embryos for successful infection under a stereo fluorescence microscope. Discard the embryos scored negative (no visible fluorescent bacteria or no visible fluorescent microspheres). Maintain embryos individually in E3 medium in 48-well plates. Refresh the medium daily.

5. Crushing of Embryos, Microscopic Scoring, and Quantitative Culture of Bacteria

1. Score all embryos microscopically for the presence of fluorescent bacteria using a stereo fluorescence microscope, starting immediately after the injections, and on each subsequent day until the embryos are randomly selected for quantitative culture.
2. Randomly select a few live infected embryos (5 to 6 in the present study) shortly after injection and transfer them individually to separate 2 mL microtubes using sterile tips. Remove the medium, wash the embryos gently with sterile PBS once and add 100 μL of sterile PBS.
3. Add 2–3 sterile zirconia beads (2 mm in diameter) to each vial and crush the embryos using the homogenizer (see **Table of Materials**) at 3,500 rpm for 30 s. Culture the homogenate quantitatively as described in step 1.3.

NOTE: Other homogenizers may require different settings.

4. Randomly select multiple embryos on subsequent days after injection for quantitative culture according to step 5.3.

6. Fluorescence Microscopy of Infection Progression and Provoked Cell Infiltration in Zebrafish Embryos

1. Anaesthetize the embryos as described in step 4.2. Pipette 500 μL of a PBS-2% (wt) methyl cellulose solution into a Petri dish containing E3 medium with 0.02% (w/v) Tricaine. Place the embryos in the methyl cellulose solution and keep them straight and horizontal.
NOTE: Methyl cellulose solution is used as a "glue", which due to its viscosity, can temporarily immobilize embryos in best orientation for imaging.
2. Use a stereo fluorescence microscope equipped with bright field, mCherry, green fluorescent protein (GFP), and UV filters to image individual embryos under identical optimized settings (e.g., intensity, gain, and exposure time) at 160x magnification. Set the focal plane such that the tissue damage caused by the injection is in focus, using the bright field filter. Set the Z-stack depth at 10 μm and step size at 5 μm , which allows for recording of 3 consecutive images.
3. Image individual embryos once daily from 5 h post-injection until 2 days post-injection. Exclude dead embryos (no heartbeat or embryo partly degraded) for further imaging and analysis. Maintain embryos individually in E3 medium in 48-well plates. Refresh the medium daily.

7. Quantitative Analysis of Fluorescence Intensity of Infection Progression and Provoked Cell Infiltration Using Object J Project File "Zebrafish-Immunotest"

1. Download "Zebrafish-Immunotest" and the detailed manual from the link: <<https://sils.fnwi.uva.nl/bcb/objectj/examples/zebrafish/MD/zebrafish-immunotest.html>>, as described in a previous study³³. In brief, open images for analysis with "Zebrafish-Immunotest", operating

under the freeware program Image J. In each loaded image, manually mark the injection site based on the observed tissue damage of embryos.

NOTE: The marked site is used by "Zebrafish-Immunotest" as the center point to detect the fluorescence peak within a distance of 50 μ m. The detected fluorescence peak is then used as the center of a standardized area (diameter of 100 μ m) for fluorescence measurement.

- Click "calculation" in the Object J menu to run the fluorescent measurement for multiple channels, if applicable, automatically for all images. Export the data and analyze by appropriate statistic test methods.

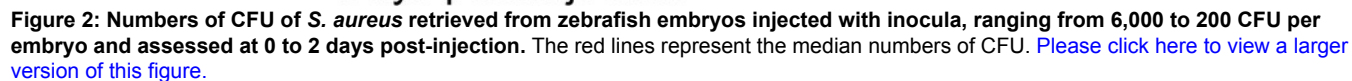
Representative Results

The present study assessed the applicability of zebrafish embryos as a novel vertebrate animal model for investigating biomaterial-associated infection. Microinjection technique has been commonly used to inject different bacterial species into zebrafish embryos to cause infection^{22,26,27,30,36}. Using the procedure depicted in **Figure 1**, *S. aureus* alone or together with 10 μ m PS microspheres (PS₁₀) were injected into the muscle tissue of zebrafish embryos in the present study. Intramuscular injection of *S. aureus* caused dose-dependent infection in embryos (**Figure 2**). The doses injected were close to the aimed challenge doses (Day 0 in **Figure 2**). Only minor variations within groups were observed. Embryos injected with high challenge doses showed variable infection progression at 1 day and 2 days post-injection (**Figure 2**, 6000 and 2000 CFU). The injected *S. aureus* bacteria either were eradicated or had proliferated and subsequently established high levels of infection within the embryos. This is similar to the reported progression of *S. aureus* infection after intravenous injection into zebrafish embryos²⁶. Embryos injected with low challenge doses of *S. aureus* cleared the bacteria (**Figure 2**, 600 and 200 CFU). These results indicated that the challenge dose of *S. aureus* strongly influences their infection progression in zebrafish embryos.

Co-injection of bacteria and microspheres resulted in higher numbers of bacteria injected than injection of bacteria only (data not shown). By preparing the bacterial inoculum for the "Bacteria-only" injections at a higher concentration (approximately 1.5 times higher) than in the Bacteria-Microspheres suspension, we were able to overcome the difference in numbers of CFU injected. This way, it is possible to inject similar numbers of bacteria into embryos in the presence and in the absence of PS₁₀ (day 0, **Figure 3**). The numbers of microspheres per injection varied. As an example, in one of our experiments, half of the embryos received 1 to 2 microspheres per injection (12 out of 25 embryos in the *S. aureus* + PS₁₀ group), some received 3 to 4 (8 out of 25 embryos), and a few received 5 to 7 microspheres (5 out of 25 embryos). However, such variations did not influence the levels of the provoked cellular responses, as reported in a previous study³³.

Next, we investigated whether the presence of microspheres has an impact on the infection progression in zebrafish embryos challenged with low doses of *S. aureus*. To this end, we injected approximately 600 CFU of *S. aureus*-mCherry with or without PS₁₀. We used two methods to study the infection progression: (i) conventional quantitative culture of the infected embryos after crushing, and (ii) scoring of microscopic detection ("microscopic scoring") of fluorescent bacteria (*S. aureus*-mCherry). We compared the results from these two methods. The score was defined as "yes or no" visibility of fluorescent bacteria in the embryos, regardless of the fluorescence intensity. Our results showed that all embryos with more than 20 CFU of *S. aureus*-mCherry were positive in microscopic scoring (red dots in **Figure 3**). For 600 CFU of *S. aureus* per embryo, the presence of PS₁₀ seemed not to significantly influence the infection progression. At all the time points, both the frequency of infected embryos (indicated on top of **Figure 3**, low challenge dose) and the numbers of CFU after crushing were not different for embryos with or without PS₁₀ (**Figure 3**, low challenge dose). This suggested that higher challenge doses of *S. aureus* are required for studying the potential effects of biomaterials on infection progression in zebrafish embryos. Hence, we injected approximately 1,000 CFU of *S. aureus* in the presence and absence of PS₁₀ into embryos. The microscopic scoring did not show differences in frequency of the infected embryos with and without PS₁₀ at any time point (**Figure 3**, high challenge dose). The quantitative culture, however, showed higher CFU numbers retrieved from embryos in the *S. aureus* + PS₁₀ group than those retrieved from the *S. aureus*-only group at 2 days post-injection (**Figure 3**, high challenge dose).

To quantify the extent of infection in the presence and in the absence of biomaterials in zebrafish embryos by image analysis, we recorded a series of images showing the infection progression of *S. aureus*-mCherry (1,000 CFU per embryo) in the presence and absence of PS₁₀ in live embryos over time (**Figure 4A**). We also recorded the provoked infiltration of Kaede green fluorescent protein-expressing macrophages in the embryos to quantify the immune cell response (**Figure 4B**). Kaede fluorescent protein may undergo color conversion from green to red under exposure to UV light, depending on the exposure time and light intensity³⁹. However, such color conversion was not observed under the experimental condition used in the present study. The fluorescence intensity of the infecting bacteria as well as of the infiltrating macrophages within a standardized area around the injection site was measured by our ObjectJ project file "Zebrafish-Immunotest", operating within Image J. "Zebrafish-Immunotest" defines a standardized area with a diameter of 100 μ m (yellow circles in **Figure 4**) around an indicated point of injection. In the embryos, this area included the majority of the fluorescent bacteria residing in the proximity of the injected microspheres, as well as the infiltrating macrophages. The presence of a biomaterial was shown to influence both initial susceptibility to infection and immune cell responses. At 5 h post-injection, macrophage infiltration in response to *S. aureus* only was significantly higher than in response to *S. aureus* + PS₁₀ (**Figure 5**, macrophage infiltration, 5 hpi). At 1 day post-injection embryos with PS₁₀ showed significantly higher levels of *S. aureus* infection than embryos without PS₁₀ (**Figure 5**, infection progression, 1 dpi). Thus, these quantitative results demonstrated that the combination of fluorescence image recording and the ObjectJ project file "Zebrafish-Immunotest" can be used to quantify the infection progression and provoked immune cell response in the presence of a biomaterial in the zebrafish embryo model. The model allows for assessing small differences in immune cell responses and levels of bacterial infection in vivo, and it only requires a stereo fluorescence microscope (with image recording) and the open-access "Zebrafish-Immunotest" for evaluation.



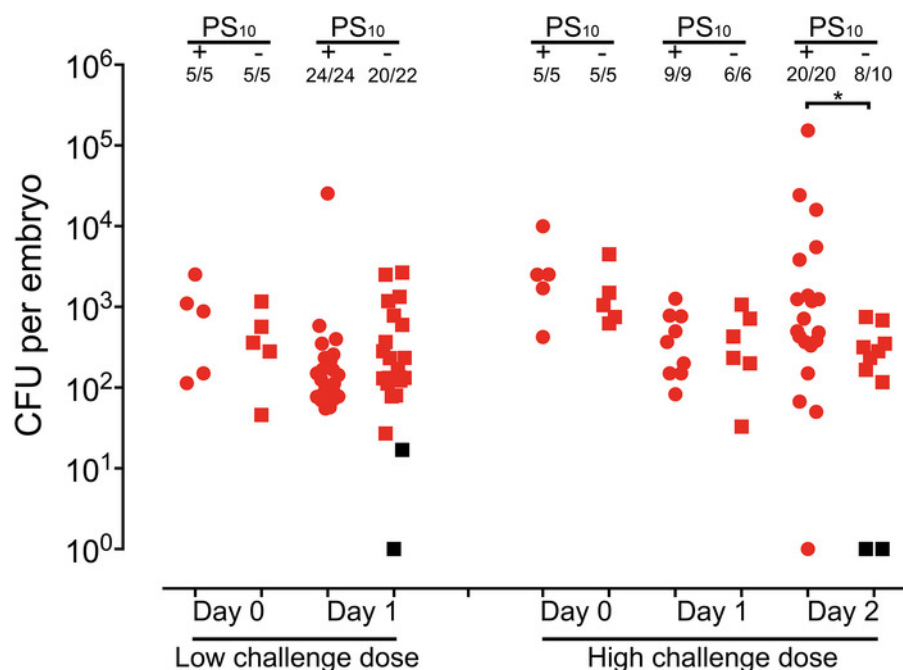


Figure 3: Numbers of CFU and microscopic scoring of mCherry protein-expressing *S. aureus* (*S. aureus*-mCherry) in zebrafish embryos, assessed at different time points. Low challenge dose: 600 CFU per embryo; High challenge dose: 1,000 CFU per embryo; PS₁₀: 10 μ m PS microspheres; "+", embryos injected with *S. aureus*-mCherry together with PS₁₀; "-", embryos injected with *S. aureus*-mCherry only, so without PS₁₀. Embryos were microscopically scored for fluorescent bacteria and randomly selected for quantitative culture of bacteria after crushing of embryos at the day of injection (Day 0) and at Day 1 and Day 2 post-injection. The embryos microscopically scored positive and negative for fluorescent bacteria are shown in red and black dots, respectively. The numbers of microscopically positive-scoring embryos divided by the total numbers of embryos scored (frequency of infected embryos) at each time point are indicated at the top of the graph. Differences in frequencies of infected embryos and in numbers of CFU between the *S. aureus* + PS₁₀ and *S. aureus* only groups at each time point were analyzed by the Fisher's exact test and Mann-Whitney test, respectively. * $p \leq 0.05$. [Please click here to view a larger version of this figure.](#)

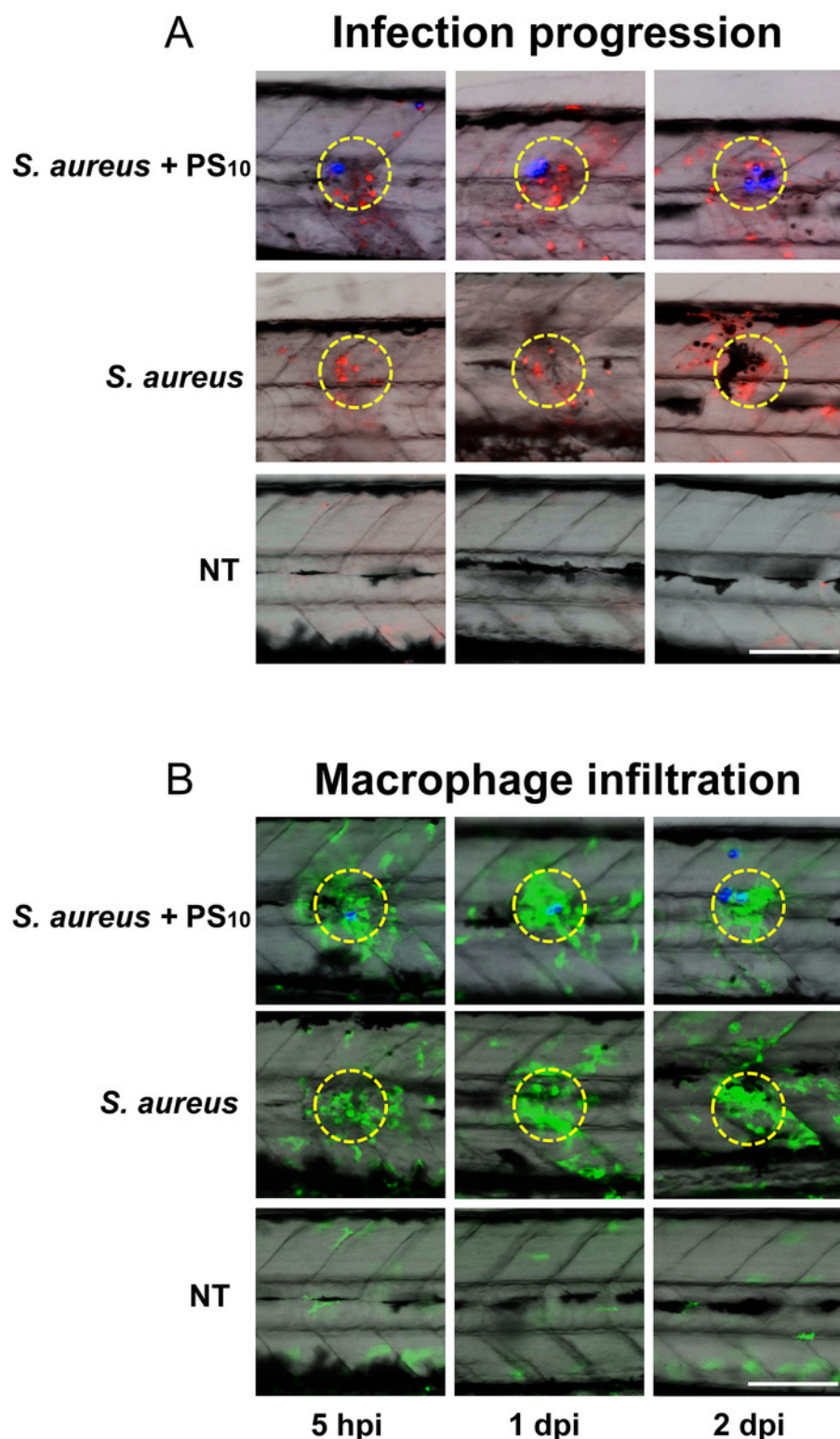


Figure 4: Representative images recording the infection progression of mCherry-expressing *S. aureus* (A, red) in the presence and absence of 10 μ m PS microspheres (PS₁₀, blue) and the provoked infiltration of Kaede protein-expressing macrophages (B, green) in embryos, from 5 h post-injection (hpi) to 2 days post-injection (dpi). Non-treated embryos (NT) were used as controls. The yellow circles (100 μ m in diameter) indicate the standardized area at the injection site for fluorescence quantification using the ObjectJ project file "Zebrafish-Immunotest". The quantified fluorescence of bacteria and of macrophages is depicted in **Figure 5**. Scale bars = 100 μ m. [Please click here to view a larger version of this figure.](#)

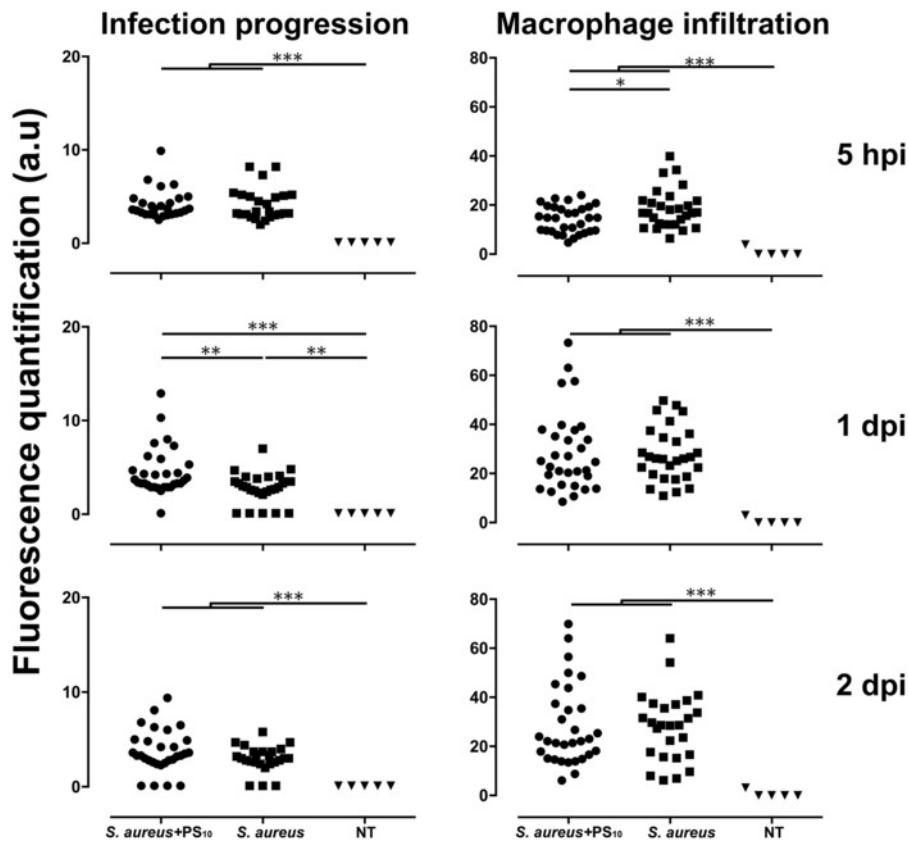


Figure 5: Fluorescence quantification of *S. aureus* infection progression and the provoked macrophage infiltration in the presence and absence of 10 μ m PS microspheres (PS₁₀) in zebrafish embryos from 5 hours post-injection (hpi) to 2 days post-injection (dpi), using the ObjectJ project file "Zebrafish-Immunotest". A standardized area at the injection site was used for fluorescence quantification (with a diameter of 100 μ m, indicated as the yellow circles in **Figure 4**). Non-treated embryos (NT) were used as controls. Differences between each two groups at each time point were analyzed by the Mann-Whitney test, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$. [Please click here to view a larger version of this figure.](#)

Discussion

Biomaterial-associated infection (BAI) is a serious clinical complication. A better understanding of the pathogenesis of BAI *in vivo* would provide new insights to improve the prevention and treatment of BAI. However, current experimental BAI animal models such as murine models are costly, labor-intensive, and require specialized personnel trained in complex surgical techniques. Therefore, these models are not suitable for high throughput analysis. Since requirements for zebrafish embryo models are less complex and costs in general are lower than for murine models, the present study assessed whether zebrafish embryos can be used as a novel vertebrate animal model for investigating BAI *in vivo*, complementary to the mammalian models.

In order to set up the zebrafish embryo BAI model, we developed a protocol of co-injection of bacteria and microspheres based on a method for intramuscular injection of microspheres into embryos described before³³. In this way, most of the bacteria are present in the vicinity of the microspheres in embryos, mimicking the bacteria-biomaterial interaction that takes place during or shortly after the implantation of biomaterials in humans/mammals.

To be able to compare infection progression in the presence and in the absence of microspheres in zebrafish embryos, embryos should be challenged with similar initial numbers of bacteria with and without microspheres. However, according to our experience, co-injection of Bacteria-Microspheres suspension results in more bacteria being injected than injection of "Bacteria-only" suspension. To address this issue, the ratio between the concentration of bacteria in "Bacteria-only" suspensions (without microspheres) and in Bacteria-microspheres suspensions should be tailored. In the present study, the concentration of *S. aureus* in the "Bacteria-only" suspension needed to be approximately 1.5 times higher than the concentration in the Bacteria-Microspheres suspension. Whether this ratio also applies to other bacterial species and other biomaterials remains to be tested. Of note, to inject similar numbers of bacteria with and without microspheres, the opening of the needles used for injections of either suspension should be as close to identical as possible.

Several properties of a biomaterial such as shape and size play an important role in determining its injectability and co-injection with bacteria. In the present study, we used 10 μ m polystyrene microspheres (PS₁₀) as model biomaterials. According to our experience, microspheres of a size of up to 15 μ m are injectable for 3 days old embryos following the protocol developed in the present study³³. For microspheres of a relatively large size (e.g., $\geq 10 \mu$ m), we discourage the use of non-monodispersed or irregularly shaped microspheres/microparticles which tend to cause clogging of the needle. If biomaterials in other shapes than round and in different sizes are chosen to be used, their injectability needs to be

tested. For (co-)injection of microspheres and bacteria, we used a 4% polyvinylpyrrolidone (PVP) solution to facilitate dispersion. This may be helpful for injection of biomaterials with and without bacteria into embryos in general.

As was the case for injections of microspheres only^{33,40}, the numbers of microspheres injected together with bacteria into embryos varied per injection, mostly ranging from 1 to 4 microspheres per embryo. However, since such variations did not result in differences in levels of the provoked immune cell response in embryos³³, they were also not expected to influence the potential effects of microspheres on infection progression. Nevertheless, novel approaches allowing injections of a single microsphere (alone or together with bacteria) would be desired.

We assessed whether microscopic scoring of presence of fluorescent bacteria can be used as a "yes or no" scoring system to analyze infection progression in live embryos. The criterion of a positive microscopic score was defined as the presence of visible fluorescent bacteria, regardless of the fluorescence intensity. Compared to quantitative culture of bacteria, our representative results suggested that microscopic scoring can distinguish embryos with 20 or more CFU of *S. aureus* bacteria from embryos with lower numbers of CFU or no infection. Thus, since only low numbers of bacteria are required for a positive score, this method is almost as sensitive as quantitative culture. Since the embryos do not need to be sacrificed and no high-end microscopes or sorting systems for fluorescence are required to perform analyses using this method, we consider microscopic scoring a simple but reliable method to monitor the infection progression of bacteria in live embryos. For quantitative analysis of the level of infection (rather than the "yes or no" scoring system as described above) and immune cell response in single live embryos, we applied the ObjectJ project file "Zebrafish-Immunotest" to quantify fluorescence intensity of the fluorescent bacteria and fluorescent macrophages recorded over time. We used a standardized area (with a diameter of 100 μ m) surrounding the injection site to measure fluorescence, since this area was shown to include a majority of the bacteria and the infiltrating macrophages. A detailed manual of "Zebrafish-Immunotest" was published previously^{33,40}. Of note, "Zebrafish-Immunotest" is an open access plug-in for ImageJ, which can be tailored by the user. For instance, the diameter of the area of analysis and other parameters of "Zebrafish-Immunotest" can be freely changed according to the study setup (see examples in the link provided in step 7 in the Protocol).

In order to show the possible infection-enhancing effects of biomaterials in zebrafish embryos, the challenge dose of bacteria needs to be assessed. In the present study, we found that a challenge dose of 1,000 CFU of *S. aureus* per embryo or higher is required. Higher levels of *S. aureus* infection in embryos with PS₁₀ than in those without PS₁₀ were found on the first 2 days after injection, indicating that the presence of biomaterials transiently facilitates outgrowth of *S. aureus* in the embryos. Whether infection remains at high levels in presence of biomaterials at later time points should be studied using older embryos under ethical approval according to applicable regulations. Since the clearance of *S. aureus* in zebrafish embryos is mainly dependent on phagocytosis and killing by macrophages and neutrophils^{23,24}, the outgrowth of bacteria might be explained by a reduced effectiveness of the phagocytes to kill the bacteria due to the presence of biomaterials. This is in line with the well-known infection-risk enhancement by biomaterials in patients^{2,4,15}, also commonly observed in more complex animal models such as mouse and other animal models^{10,11,12,13}. In addition to *S. aureus* infection, infection progression of *S. epidermidis* or other pathogens in the presence of biomaterials can be investigated following the protocol described in the present study. From the point of biomaterials, several material properties (e.g., chemical composition, hydrophobicity, roughness, and surface charges) may influence the cellular responses and/or bacteria-material interaction^{41,42}. Our previous study has shown that injection of a biomaterial (in the presence of PVP) provoked a stronger macrophage infiltration compared to injection of only PVP in zebrafish embryos. Moreover, zebrafish embryos reacted differently to microspheres made of poly (ϵ -caprolactone) and polystyrene³³. Therefore, injection of microspheres of different nature may also have differential effects on enhancement of infection, which can be relatively easily assessed by the method described here.

For further development, this zebrafish embryo BAI model may be amended for a high throughput system featuring automated robotic injection^{27,43}, complex object parametric analysis and sorting (COPAS) systems, and high throughput RNA sequence analysis^{27,44}. Such zebrafish embryo-based high throughput BAI models may be used as whole animal systems for *in vivo* screening and testing of (novel) anti-infective biomaterials as well as testing of the efficacy of antibiotic treatments or other anti-BAI strategies. Moreover, intracellular survival is one major strategy of bacteria to survive in the presence of biomaterials^{9,10,11,12,13}. The usefulness of zebrafish embryos for studying intracellular infection has been shown in several studies^{36,46}. Thus, our embryo model may be used to study the intracellular survival of staphylococci or other intracellular pathogens in the presence of biomaterials, and strategies to treat such intracellular infections. In addition, *in situ* hybridization techniques⁴⁵ can be used in the model to study the influence of bacteria or biomaterials or their combination on the expression of particular genes at the injection site, which may be helpful to discover marker genes for BAI.

In summary, the present study shows the potential of zebrafish embryos with the methods developed here to study biomaterial-associated infection in real time *in vivo*. This zebrafish embryo model may therefore be used to investigate novel infection-resistant biomaterials and promising prevention and treatment strategies of BAI, and to close the gap between *in vitro* studies and larger animal models.

Disclosures

The authors have nothing to disclose.

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