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TITLE:

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

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KEYWORDS:

Zebrafish embryos, biomaterial-associated infection, *Staphylococcus aureus*, polymeric microspheres, *in vivo* visualization, intravital analysis, fluorescence quantification

SUMMARY:

The present study describes a zebrafish embryo model for *in vivo* visualization and intravital analysis of biomaterial-associated infection over time based on fluorescence microscopy. This model is a promising system complementing mammalian animal models such as mouse models for studying biomaterial-associated infections *in vivo*.

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 (PS_{10}) 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 PS_{10} 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.

INTRODUCTION:

A variety of medical devices (referred to as "biomaterials") are increasingly used in modern medicine to restore or replace human body parts[1]. 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[2-6]. 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 biomaterial[7]. 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 bacteria[8]. Thus, the presence of biomaterials can facilitate intracellular survival of bacteria[9-13] 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 strategies[18]. 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[18-21]. However, this technique is relatively complex and still in its infancy, and several issues must be addressed for quantitative analysis of BAI[18]. 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 mycobacteria[22], *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 macrophages[32] 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 feasible[33]. 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 microspheres[33]. 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, zebrafish embryos are 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.

1.1. 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.

1.1.1. 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 (OD_{620}). Culture the bacteria until the OD_{620} reaches 0.4-0.8.

Note: An OD_{620} of 0.1 generally corresponds to 3.0 x 10^7 CFU/mL *S. aureus*. The OD_{620} 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.

1.2. Centrifuge bacteria at 3500 x 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) polyvinylpyrrolidone$_{40}$ (PVP_{40}) solution in PBS.

1.3. Vortex this bacterial suspension and dilute 100 µL of the suspension in 900 µL of sterile PBS in a cuvette for the OD_{620} measurement. Adjust the concentration of the bacterial suspension with PVP_{40} solution. Check by quantitative culture of 10-fold serial dilutions as below.

1.3.1. Transfer 100 µL of the bacterial suspension 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 mannitol salt agar-2 (MSA-2) plates, incubate the plates at 37 °C overnight, count the colonies, and calculate the numbers of bacteria (CFU).

1.4. Centrifuge the commercial polystyrene (PS) microspheres (blue fluorescent, 10 µm) at 1000 x g, discard the supernatant, and re-suspend the microspheres 1:1 in bacterial suspension in PVP_{40} solution to form bacteria-microspheres suspension. Mix the suspensions by vortexing.

Note: Biomaterials can be freely chosen to mix with bacterial suspension.

1.5. In order to inject approximately equal doses of bacteria in both the presence and absence of microspheres, adjust the concentration of the "bacteria-only" suspension (without microspheres) to be approximately 1.5 times higher than that of the bacteria-microspheres suspension.

Note: This ratio 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.

2. Breeding, Harvesting, and Maintenance of Zebrafish Embryos

2.1. 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 for breeding added to induce the adult females to produce eggs after the light turns on, then separate adults from the produced eggs.

2.2. 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 medium[37] and incubate at 28 °C. Remove dead and abnormal embryos, and refresh the E3 medium daily.

3. Preparation of Injection Needles

3.1. 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.

3.2. 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 survival[38].

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

4.1. 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.

4.2. 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, select embryos expressing green fluorescent proteins using a stereo fluorescence microscope.

4.3. 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.

4.3.1. 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.

4.4. 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.

4.5. 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

5.1. 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.

5.2. 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.

5.3. 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 3500 rpm for 30 s. Culture the homogenate quantitatively as described in step 1.3.

Note: Other homogenizers may require different settings.

5.4. 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

6.1. 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.

6.2. 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.

6.3. Image individual embryos once daily from 5 hours post-injection until 2 d 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"

7.1. 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 study[33]. 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 µm. The detected fluorescence peak is then used as the center of a standardized area (diameter of 100 µm) for fluorescence measurement.

7.2. 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 µm PS microspheres (PS_{10}) 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 d and 2 d 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 embryos[26]. 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 PS_{10} (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* + PS_{10} 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 study[33].

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 PS_{10}. 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 PS_{10} 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 PS_{10} (**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 1000 CFU of *S. aureus* in the presence and absence of PS_{10} into embryos. The microscopic scoring did not show differences in frequency of the infected embryos with and without PS_{10} at any time point (**Figure 3**, high challenge dose). The quantitative culture, however, showed higher CFU numbers retrieved from embryos in the *S. aureus* + PS_{10} group than those retrieved from the *S. aureus*-only group at 2 d 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 (1000 CFU per embryo) in the presence and absence of PS_{10} 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 intensity[39]. 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 µm (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* + PS_{10} (**Figure 5**, macrophage infiltration, 5 hpi). At 1 d post-injection embryos with PS_{10} showed significantly higher levels of *S. aureus* infection than embryos without PS_{10} (**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 AND TABLE LEGENDS:

Figure 1: Schematic procedure of co-injection of bacteria-microspheres suspension into the tail muscle of zebrafish embryos. This figure has been modified from Zhang *et al.*[33] with permission.

Figure 2: Numbers of CFU of *S. aureus* retrieved from zebrafish embryos injected with inocula, ranging from 6000 to 200 CFU per embryo and assessed at 0 to 2 d post-injection. The red lines represent the median numbers of CFU.

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: 1000 CFU per embryo; PS_{10}: 10 µm PS microspheres; "+", embryos injected with *S. aureus*-mCherry together with PS_{10}; "-", embryos injected with *S. aureus*-mCherry only, so without PS_{10}. 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* + PS_{10} and *S. aureus* only groups at each time point were analyzed by the Fisher' exact test and Mann-Whitney test, respectively. $*p \leq 0.05$.

Figure 4: Representative images recording the infection progression of mCherry-expressing *S. aureus* (A, red) in the presence and absence of 10 µm PS microspheres (PS_{10}, blue) and the provoked infiltration of Kaede protein-expressing macrophages (B, green) in embryos, from 5 h post-injection (hpi) to 2 d post-injection (dpi). Non-treated embryos (NT) were used as controls. The yellow circles (100 µm 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**. The scale bars represent 100 µm.

Figure 5: Fluorescence quantification of *S. aureus* infection progression and the provoked macrophage infiltration in the presence and absence of 10 µm PS microspheres (PS_{10}) 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 µm, 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, $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$.

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 before[33]. 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 µm polystyrene microspheres (PS_{10}) as model biomaterials. According to our experience, microspheres of a size of up to 15 µm are injectable for 3 days old embryos following the protocol developed in the present study[33]. For microspheres of a relatively large size (*e.g.,* ≥ 10 µm), 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 embryos[33], 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 to 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 µm) 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 1000 CFU of *S. aureus* per embryo or higher is required. Higher levels of *S. aureus* infection in embryos with PS_{10} than in those without PS_{10} 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[10-13]. 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 polystyrene[33]. 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[9-13]. 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 techniques[45] 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.

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DISCLOSURES:

The authors have nothing to disclose.

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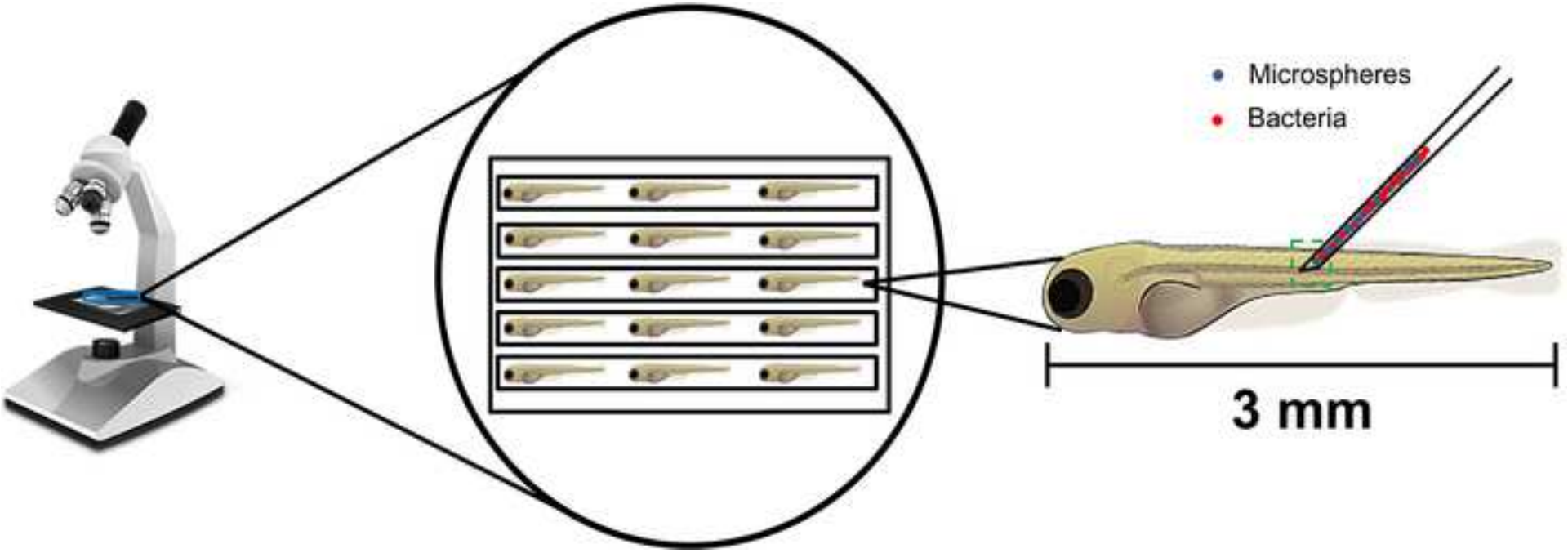
Figure 1



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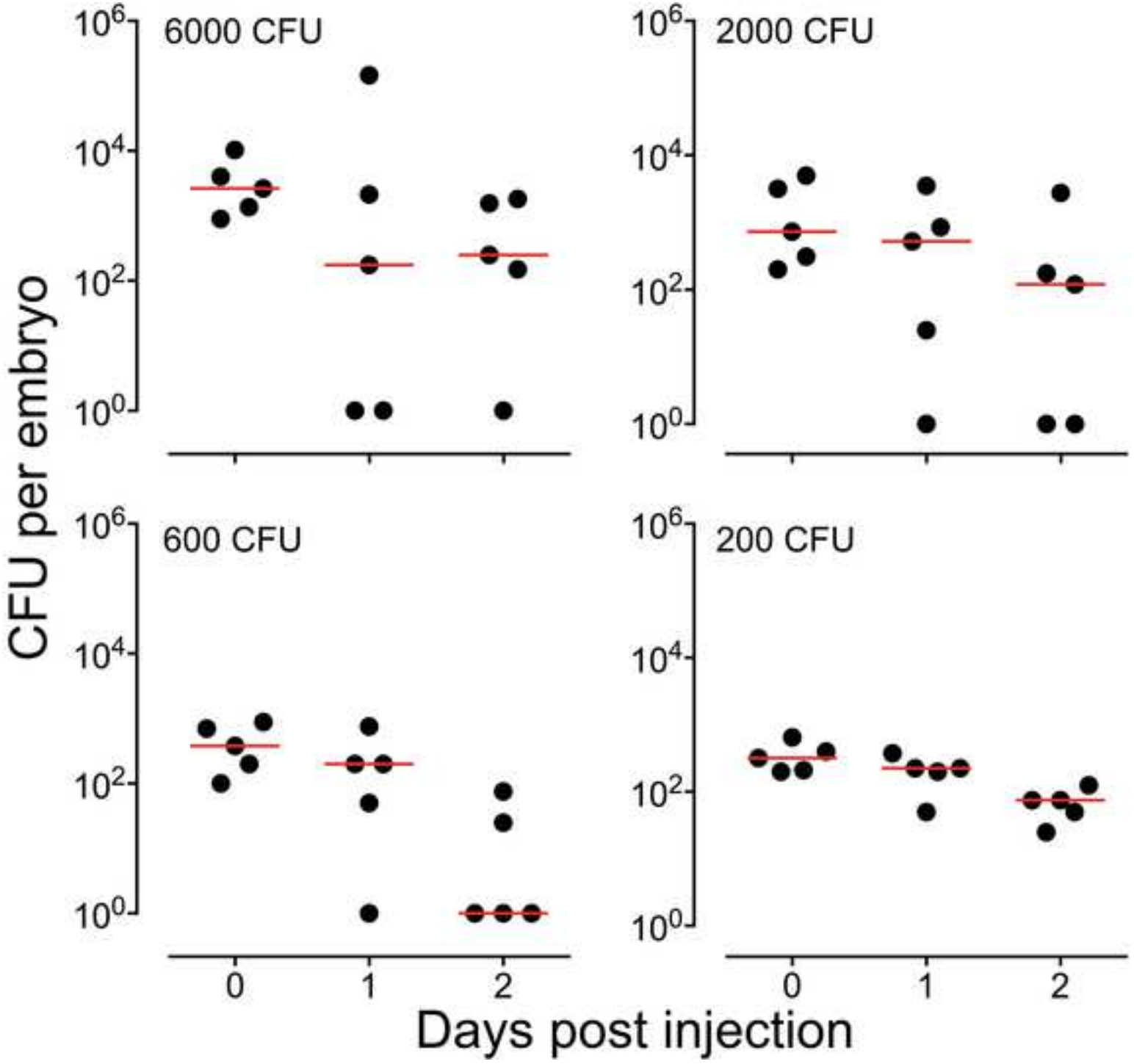

Figure 3

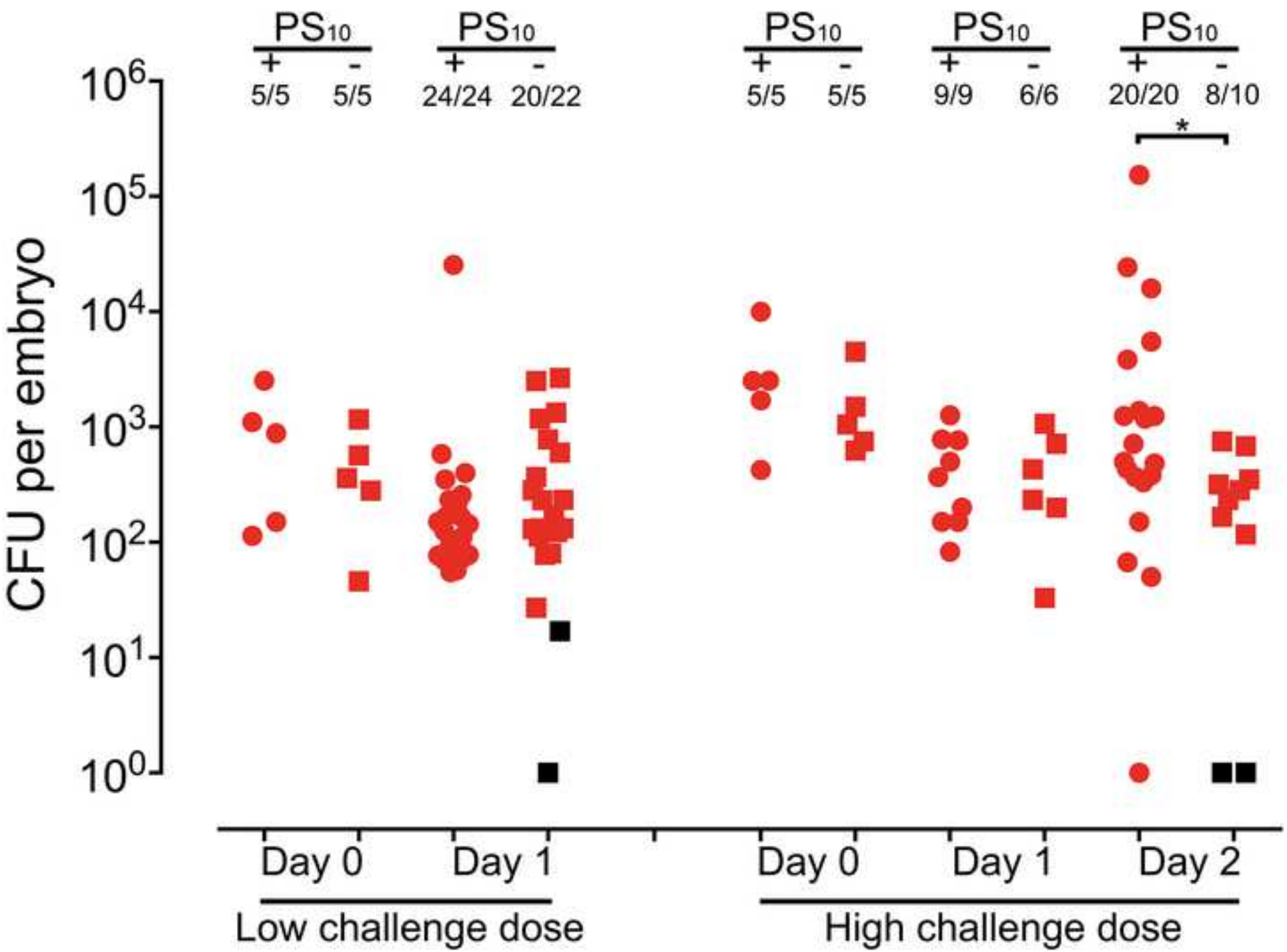

Figure 4

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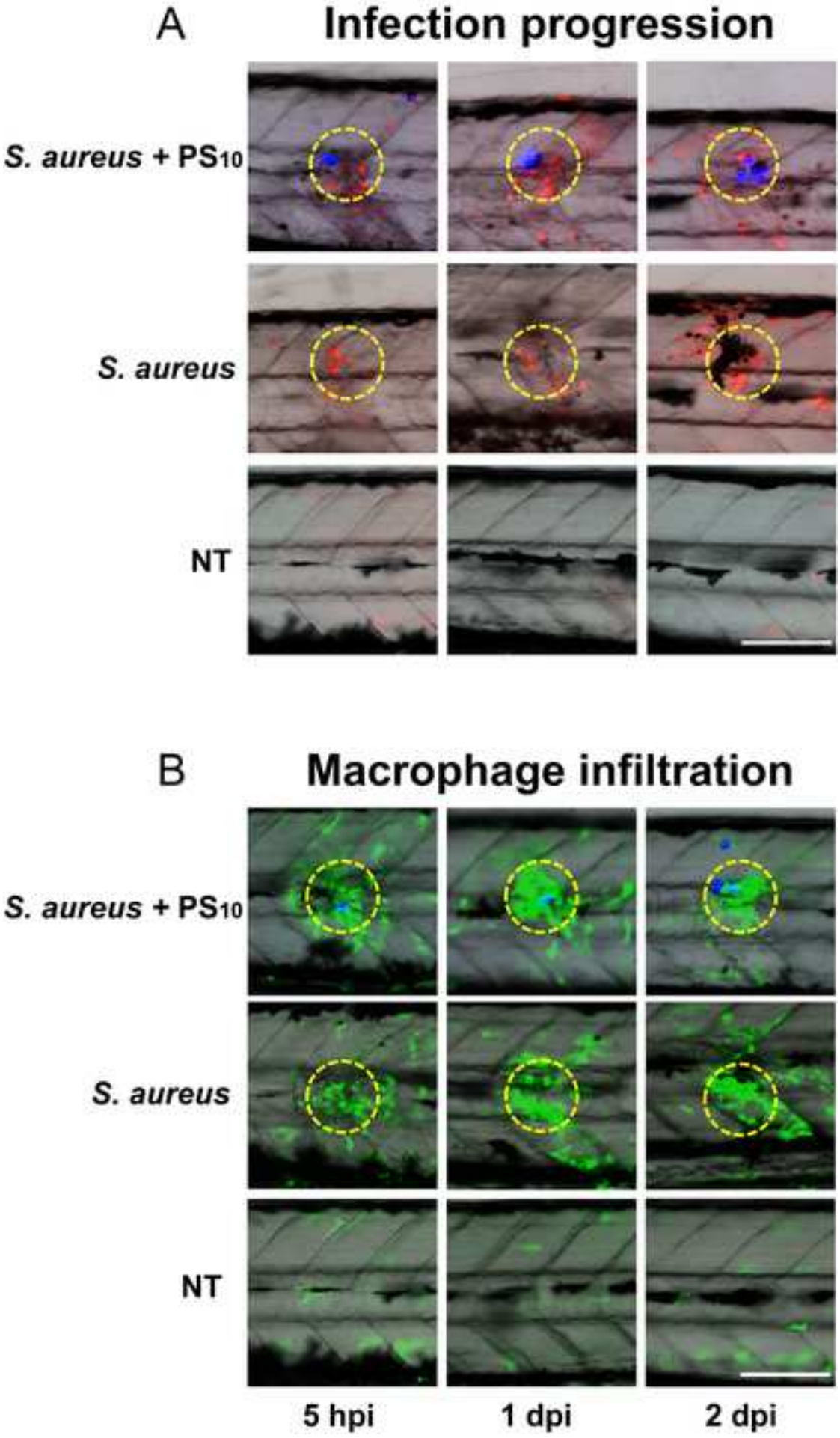
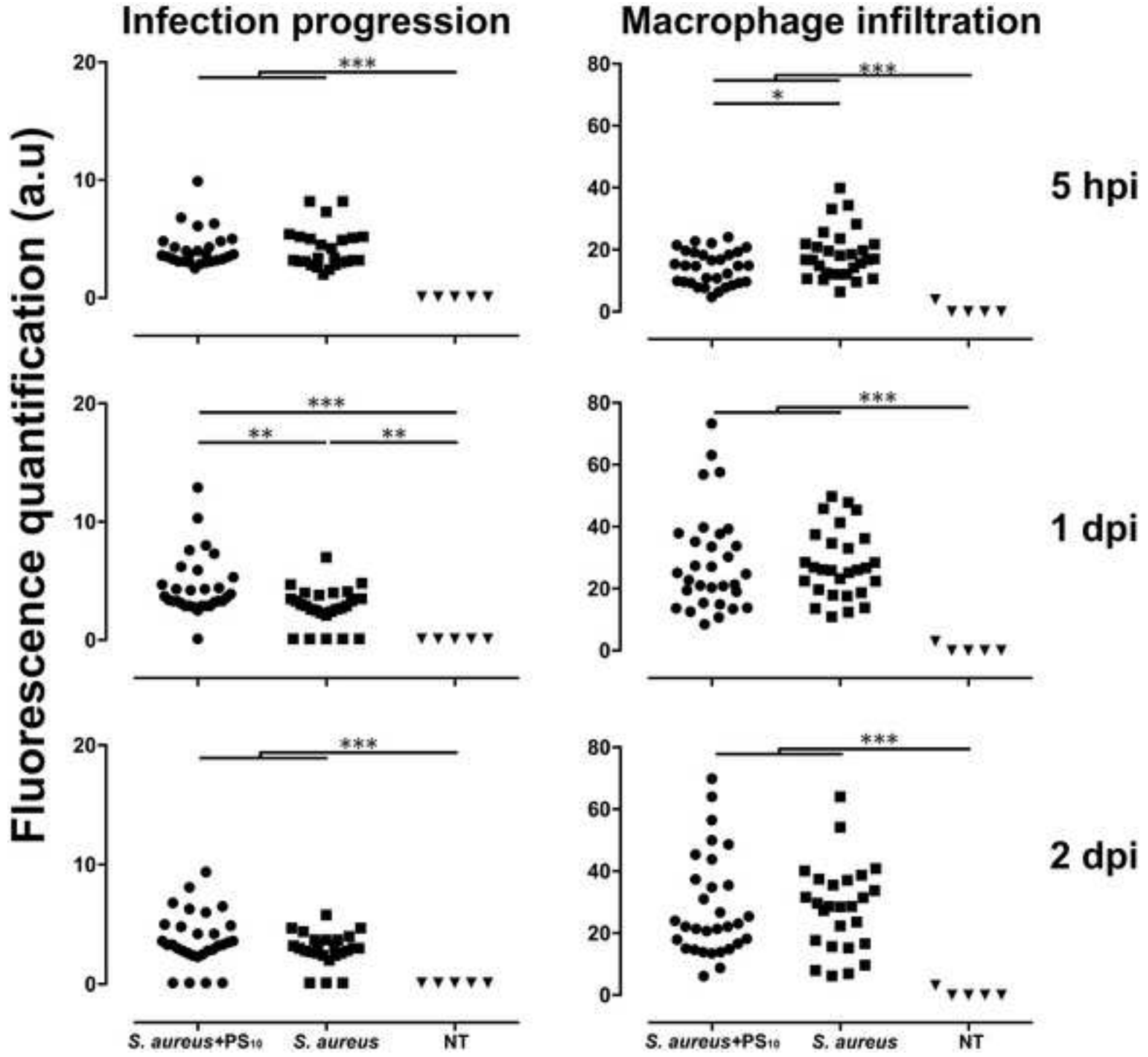


Figure 5



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Tryptic soya agar	BD Difco	236950	Media preparation unit at AMC
Tryptic soya broth	BD Difco	211825	
Polyvinylpyrrolidone40	Applichem	A2259.0250	
10 µm diameter polystyrene microspheres (blue fluorescent)	Life technology/ThemoFisher	F8829	
Glass microcapilary (1 mm O.D. x 0.78 mm	Harvard Apparatus	30-0038	
Micropipette puller instrument	Sutter Instrument Inc	Flaming p-97	
Light microscope LM 20	Leica	MDG33 10450123	
3-aminobenzoic acid (Tricaine)	Sigma-Aldrich	E10521-50G	
Agarose MP	Roche	11388991001	
Stereo fluorescent microscope LM80	Leica	MDG3610450126	
Microloader pipette tips	Eppendorf	5242956.003	
Micromanipulator M3301 with M10 stand	World Precision Instruments	00-42-101-0000	
FemtoJet express micro-injector	Eppendorf	5248ZO100329	
Microtrube 2ml pp	Sarstedt	72.693.005	
Zirconia beads	Bio-connect	11079124ZX	
MagNA lyser	Roche	41416401	
MSA-2 plates (Mannitol Salt Agar-2)	Biomerieux	43671	Chapmon 2 medium
Methyl cellulose 4000cp	Sigma-Aldrich	MO512-250G	
Chloramphenicol	Sigma-Aldrich	C0378	
Gyrotory shaker (for bacterial growth)	New Brunswick Scientific	G10	
Zebrafish incubator	VWR	Incu-line	
Cuvettes	BRAND	759015	
Centrifuge	Hettich-Zentrifugen	ROTANTA 460R	
Spectrometer	Pharmacia biotech	Ultrospec®2000	
Forceps	Sigma-Aldrich	F6521-1EA	
48 well-plates	Greiner bio-one	677180	
96 well-plates	Greiner bio-one	655161	
Petri-dish	Falcon	353003	
Petri-dish	Biomerieux	NL-132	
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Article Title: A zebrafish embryo model for in vivo visualization and intravital analysis of biomaterial-associated staphylococcus aureus infectio

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Editorial comments:

Changes to be made by the Author(s):

Question 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.

Answer: We have thoroughly proofread the manuscript, following the editor's suggestion. Changes are marked in track changes.

Question 2. Figure 1: Please include a space between numbers and their units (i.e., 3 mm).

Answer: We have included a space between numbers and their units in Figure 1, following the editor's suggestion.

Question 3. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Answer: We have used SI abbreviations when applicable, following the editor's suggestion.

Question 4. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Answer: We have included a space between all numbers and their corresponding units, following the editor's suggestion.

Question 5. Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Answer: We have moved the ethics statement before our numbered protocol steps, following the editor's suggestion.

Question 6. 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 and Reagents. For example: FemtoJet, MagNA, Image J, Prism GraphPad 7.0, etc.

Answer: We have removed all commercial language. "Image J" is an open access and non-commercial image analysis tool. We have indicated this in the excel list of materials and provide the home page for downloading the program. In 2 cases, settings of equipment are specific for the instruments used, i.e for the FemtoJet micro-injector and for the MagNA lyser homogenizer. We have indicated that the settings need to be adjusted for the device used by the users, and refer to the "Table of Materials and Reagents" for this.

Question 7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Answer: We have revised the protocol text to avoid the use of personal pronouns, following the editor's suggestion.

Question 8. 1.2: Centrifuge bacteria for how long? Please also specify the volume of PBS used in this step.

Answer: We have added the relevant information in 1.2 of the Protocol, following the editor's suggestion.

Question 9. 1.3: Please mention how to check by quantitative culture of 10-fold serial dilutions. Use sub-steps as necessary.

Answer: We have added the detail on quantitative culture of 10-fold serial dilutions in 1.3 of the Protocol.

Question 10. 1.4: Please write the text in the imperative tense. After centrifugation of microspheres, is the supernatant discarded? Please specify.

Answer: We have used imperative tense in the text in 1.4 of the Protocol, following the editor's suggestion. After centrifugation of microspheres, the supernatant is discarded. We have added this information to 1.4 of the Protocol.

Question 11. 2.3: What are considered abnormal eggs? How large is the petri-dish?

Answer: We have corrected "eggs" to "embryos" since when we collected them the eggs have already been fertilized and have become "embryos". Regarding the size of the petri-dish, We have added the information of the diameter of the petri-dish (100 mm) in 2.3 of the Protocol.

Question 12. 4.1: How large is the petri-dish? Please specify throughout.

Answer: We have specified the diameter of the petri-dish throughout the manuscript when applicable.

Question 13. 5.4: Please specify the "above steps" repeated here.

Answer: We have specified the "above steps" in 5.4 of the Protocol.

Question 14. 6.1: Please mention how embryos are anesthetized.

Answer: We have clarified how embryos are anaesthetized in 6.1 of the Protocol.

Question 15. 6.3: Please point out the specific steps for maintaining embryos.

Answer: We have pointed out the specific steps for maintaining embryos in 6.3 of the Protocol.

Question 16. Discussion: Please also discuss critical steps within the protocol and any limitations of the technique.

Answer: We have expanded the discussion on critical steps within the protocol and some limitations of our model in the Discussion (page 12, the paragraphs "to be able to compare ……", "several properties ……" and "As was the case for injections ……").

Question 17. References: Please do not abbreviate journal titles.

Answer: We have used the full title of all journals, following the editor's suggestion.

Reviewers' comments:
Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please thoroughly address each concern by revising the manuscript or addressing the comment in your rebuttal letter.

Reviewer #1:
Manuscript Summary:
The Problem of BAI is of scientific interest and the authors provide an in vivo model suitable for high throughput screening. The model combines existing zebrafish embryo models with biomaterials and S. aureus infection. The method can allow one to compare infection and immune cell activity between groups, e.g. those with versus those without biomaterials. There are many possibilities for this model. I see great value for the field.

Major Concerns:
no major concerns from written text

Minor Concerns:
Question 1: The authors state that mouse models are labor-intensive and. I would suggest the zebrafish will also require specialized equipment, personnel and training.

Answer: We agree with the reviewer that the zebrafish will also require specialized equipment, personnel and training. However, in our opinion, such requirements for zebrafish embryos are less complex than that for mouse models, and costs per material to be analysed are much lower. We have made minor changes to the sentence in the manuscript in the Discussion (page 12, in the 1st paragraph, the text marked in track changes).

Question 2: The authors state that other S. aureus strains or other bacterial species may be used. Similarly, the authors state that "Biomaterials may be freely Chosen". I would like some comment on Prior experience on each factor. If a viewer chose another species, are they likely to be able to

perform the experiment as described without any surprises? For example, if we use silicone, ceramic or metal biomaterials, what is the risk of unexpected complications in the model.

Answer: Many different bacterial species have been studied in zebrafish embryos in the literature (e.g. *S. aureus, S. epidermidis, Mycobacterium marimum, Psuedomonas aeruginosa, Escherichia coli* and *Enterococcus faecalis,* See Reference 22-27). According to our experiences so far, injection of bacterial suspension likely is possible for any bacterial species/strain. Besides the *S. aureus* strain RN4220 which we used as an example in the present study, we also have successfully injected other *S. aureus* strains and strains of other bacterial species such as *Staphylococcus epidermidis*. Therefore, from a technical point of view injections of other *S. aureus* strains or bacterial species can be performed following the protocol we provided in this manuscript. For injections of biomaterial-bacteria suspensions or biomaterials only, several characteristics of a biomaterial such as shape and size play a role in determining the injectability of the material. For instance, we also have tried to inject titanium microparticles with irregular shape, which are difficult to be dispersed in suspension. These particles caused clogging of the needle, and were very difficult to inject. Therefore, monodispersed and regularly shaped microspheres/microparticles are likely desired for injections. Biomaterials with other shapes than round may be also injectable, but this will require testing. In the present study, we used PVP to disperse the microspheres, which may be helpful for certain biomaterials but not necessary for injection of other biomaterials. Moreover, another important parameter influencing injectability of biomaterials is the size of the opening of the needle required for injections. Although we did not test this in detail, there is a maximum of the opening in order not to cause too much tissue damage by the injections. Therefore, the injectability of the biomaterial may vary significantly among biomaterials with different sizes and shapes.

We have briefly indicated this in the manuscript in the Discussion (Page 12, the 3rd and 4th paragraphs marked in track changes).

Question 3: It was not clear why you use Methyl cellulose in 6.1. Perhaps a quick Explanation is needed.

Answer: As we mentioned in 6.1, methyl cellulose solution in PBS is used to (temporarily) immobilize and keep embryos straight and horizontal during imaging recording. We have made this more clear in 6.1 of the Protocol.

Question 4: Similarly, from the text, the type of mold, and its purpose, in 4.1 is not clear.

Answer: We have clarified the type and purpose of mold mentioned in 4.1 of the Protocol.

Reviewer #2:

Manuscript Summary:

The authors describe a biomaterial associated infection (BAI) model where an mCherry-labeled Staphylococcus aureus strain is microinjected intramuscularly into 3 day post fertilization mpeg1:Kaede transgenic zebrafish larvae with or without 10um polystyrene microspheres (PS). The authors measure bacterial burden over time in larvae injected with bacteria alone or bacteria with PS and find that at a higher challenge dose of 1000 CFU, 2 days post fertilization there is a statistically

higher level of bacterial burden in fish injected with a suspension of bacteria and PS than in fish injected with bacteria alone. The authors then use fluorescent microscopy and the ObjectJ project file "Zebrafish-Immunotest" operating in Image J to quantify bacterial burden (infection progression) over time as a function of mCherry fluorescence and macrophage infiltration as a function of GFP fluorescence at the injection site. They find that there is a statistically higher level of mCherry fluorescence at 1 dpi, though not at 5 hpi or 2 dpi, in larvae injected with bacteria and PS compared with larvae injected with bacteria alone. They also find that there is a statistically different increase in macrophage infiltration to the site of infection 5 hpi in larvae injected with bacteria alone compared to larvae injected with bacteria and PS. The authors suggest that their model will be of use in studying BAI infection in real time in vivo.

Major Concerns:

Question 1: The rationale for developing a BAI model in zebrafish embryos/larvae is strong and such a model would be incredibly useful. It was therefore disappointing to see that the robustness of the assay does not appear to be great.

Answer: The purpose of this manuscript is to provide a protocol for a novel zebrafish embryo biomaterial-associated infection (BAI) model. In the present study we used the *S. aureus* strain RN 4220 and polystyrene (PS) microspheres as bacterium and biomaterial, respectively, to develop this model. The comments of Reviewer#2 are focused very strongly on the outcomes of this example experiment, whereas we have focused particularly on reporting a novel method, and therefore specifically chose JOVE for its dissemination. The results of our experiment with *S. aureus* in combination with PS microspheres only represent one example interaction. Responses to other combinations of bacteria and materials may be quite different, which can now be assessed by our novel embryo model.

Contrary to the view of the reviewer we *do* consider our model robust, since in the present study it allowed detection of subtle differences in susceptibility to infection. The numbers of zebrafish embryos in principle is unlimited, allowing detection of small/subtle differences which may not be detected using e.g. mouse models which have to use lower numbers of animals.

Question 2: The gold standard for assaying bacterial colonization in any organism is CFU determinations. In the BAI model described, the authors show that only at 2 days post infection is there a statistically different increase in bacterial colonization in larvae injected with bacteria and PS compared with bacteria alone. While this difference is significant, it is a result of a really wide spread in the data of CFU determinations in fish injected with bacteria and PS, such that it appears that only 25% of larvae injected with bacteria and PS actually have a higher burden than larvae injected with bacteria alone. In other words, three quarters of the cohort injected with bacteria and PS is responding as if it was injected with bacteria alone.

Answer: As the reviewer agrees, a significant difference was observed based on CFU determinations in the present study. As the numbers of bacteria per embryo were not always normally distributed, we used non-parametric statistics. This is a common approach in biomaterial infection studies using other animal models as well (See Reference 10, 12 and 13). Thus, as described in the caption of Figure 3 the

statistical analysis of the corresponding data is based on Mann-Whitney testing which compares the differences in the *ranks* of individual data points (CFU numbers), so *not* the actual CFU numbers. The analysis showed that embryos/larvae injected with bacteria and PS had statistically significantly higher CFU burden than embryos injected with only bacteria at 2 days post infection.

Question 3: Part of the utility of the method described is the fluorescent quantitation of macrophage and bacterial cell numbers and interactions between these cells that is possible using transparent larvae with fluorescently labeled macrophages and bacteria. While it is highly desirable to use fluorescence (in this case mCherry) as a surrogate for bacterial burden, the experiment described in Fig. 5 simply isn't concordant with the CFU determinations displayed in Fig. 3. In other words, if there is a statistical difference in bacterial burden by CFU in larvae injected with bacteria and PS at 2 dpi, one would expect there to be an increase in fluorescence quantified at 2dpi in Figure 5 but there isn't, though there is a difference at 1 dpi. Since the data is discordant, it calls into question both the robustness of the assay and whether mCherry fluorescence is really a reliable surrogate for bacterial burden here.

Answer: We have shown earlier that for the closely related *S. epidermidis* carrying the identical mCherry plasmid, fluorescence and numbers of CFU show a good correlation (Reference 27). We assume that this also holds true for the *S aureus* strain since it carries the same plasmid and fluorescence intensity of the mCherry-expressing *S aureus* and *S epidermidis* strains is highly similar.

Fig.5 and Fig. 3 show the results of two different experiments. In both experiments there are transiently higher levels of *S. aureus* infection in the presence of PS microspheres, although at different time points. Minor differences in conditions between the experiments (different batches of embryos and injections of bacteria) might have caused the observed difference in the moment of transiently increased susceptibility.

Question 4: Finally, while the authors do show a statistical difference in macrophage infiltration 5 hpi between larvae injected with bacteria and PS and bacteria alone, the difference in macrophage numbers infiltrating is scant (addressing the mean numbers of infiltrating macrophages would be useful here). Again, it would appear that only a small fraction of the cohort injected with bacteria alone is responding differently from the cohort injected with bacteria and PS, suggesting that by this measurement the assay simply isn't very robust. While a BAI model in zebrafish embryos/larvae would be incredibly useful, the lack of robustness in the assay makes the current model difficult to embrace.

Answer: As the reviewer agrees, a significant difference in macrophage infiltration was observed at 5 hpi in the present study. The significance of this difference has been assessed using Mann-Whitney test analysis. This is the appropriate statistical approach, as argued in our answers to Question 2 of Reviewer#2. Counting the number of infiltrating macrophages in the zebrafish embryo is technically not possible due to accumulation and “overlapping” of these cells in the images (as shown in Fig.4). The *S. aureus* strain RN4220 used as an example in the present study is an accessory gene regulator A (*agrA*) mutant strain. This strain was chosen because of its high transformability allowing easy introduction of the plasmid carrying the gene encoding the mCherry protein. The strain has been reported to have reduced virulence in other animal models (Reference 34). It is therefore possible that

the strain even in the presence of biomaterials cannot establish severe infection in the embryos and provoke strong macrophage infiltration. However, we think that the fact that minor differences can be assessed in the zebrafish embryo BAI model actually supports the robustness of the model.

Reviewer #3:

Manuscript Summary:

The authors have developed a model and describe an application of its use to study biomaterial-associated infections (BAI). This is a serious clinical issue and research in this area is warranted. The authors use zebrafish embryos to study BAI, which allows for in vivo visualization of host-pathogen-biomaterial interactions and high-throughput screening. This zebrafish BAI model will advance our understanding of BAI by allowing researchers to ask questions and make observations that are not accessible when using other animal models of BAI. I have listed my minor concerns to hopefully improve the manuscript below.

Minor Concerns:

Question 1: Please describe what is currently known about the role of macrophages in BAI and/or Staph infections in the introduction.

Answer: We have modified the text describing the role of macrophages in BAI in the Introduction (page 1, the 1st paragraph, the text marked in track changes).

Question 2: Macrophage infiltration in response to injection of the biomaterial alone vs an appropriate control seems like a missing experimental condition to me. Was this done in reference 31? If so, please state the results of that experiment somewhere in this manuscript.

Answer: In reference 31, we studied macrophage infiltration in response to injection of the biomaterial (in presence of PVP) and to control injection of PVP. The results show that injection of the biomaterial provoked a prolonged macrophage infiltration compared to injection of PVP. We have added a statement regarding this result in the Discussion in this manuscript (page 12, line 16-17).

Question 3: Do injectable biomaterials come in shapes other than solid microspheres? Would an option with a hollow center be more representative of what is implanted into patients in the clinic?

Answer: As we mentioned in our answer to Question 2 of Reviewer#1, characteristics such as shape and size may influence the injectability of biomaterials for zebrafish embryos. Biomaterials in shapes other than solid microspheres may also be injectable. However, in our experience monodispersed and regularly shaped microspheres are much easier to inject than non-monodispersed and/or irregularly shaped ones which tend to cause clogging of the needle. This may also be true for biomaterials in other shapes.

In the present study, we used microspheres as model biomaterials, whether the option with a hollow center or other types of biomaterials are injectable to zebrafish embryos needs to be tested. We do not think that a hollow material is more relevant to the clinic, since the embryo model is not suited to assess anything like a catheter function. The model is aimed to test biomaterials for immune response induction and infection susceptibility as such. The results may subsequently guide the choice of materials suited for manufacturing of specific medical devices.

Question 4: I am confused how a bacterial solution that has its concentration cut in half by the addition of microspheres can result in more bacteria being injected vs injecting the undiluted bacterial solution if the same volume is being injected. Can you explain this finding?

Answer: The reviewer is rightly confused. We do not really understand why more bacteria are injected when microspheres are present. One hypothesis would be that the fluid dynamics at the injection site are influenced by the bead. Another hypothesis would be that bacteria may adhere to the surface of microspheres and are injected as "extra" bacteria carried by the microspheres. The method as described however corrected the situation, allowing approximately identical numbers of bacteria to be injected with or without microspheres.

Question 5: Would an alternate experimental design be to inject the microspheres vs an appropriate control solution first and then at a later time point inject the same volume of the same bacterial solution into the same area?

Answer: For 2 reasons we think it is better to use the co-injection approach. First, we aimed to minimize the number of injections since embryos receiving multiple injections are at higher risks to die than the ones receiving single injection. Secondly, in real cases of biomaterial associated infection, bacteria in most case will enter the patient together with the implant, attach to the surface of biomaterials and cause infections. As we stated in the Discussion (page 12, in the 2nd paragraph, the sentence " in this way, most of ……"), co-injection of biomaterial and bacteria therefore mimics the interactions between biomaterial and bacteria during implantation of biomaterial.

Question 6: Please add references after "possession of an immune system highly similar to that of mammals" in the introduction.

Answer: We have added references (Reference 28 and 29) to support this statement, following the reviewer's suggestion.

Question 7: Please state how you determine mid logarithmic phase growth and how long you centrifuge at 3500xg to pellet the bacteria.

Answer: We have added the relevant information in 1.1 and 1.2 of the protocol.

Question 8: Please provide a reference (another JoVE video perhaps) that details the breeding and collecting of zebrafish embryos from spawnings of adult zebrafish.

Answer: We have added new references regarding the detail of the breeding and collection of zebrafish embryos (References 35 and 36), following the reviewer's suggestion. Of note, the procedure we used to breed and collect zebrafish embryos are not entirely same to the procedure

described in the reference.

Question 9: Please define the acronyms TL and MSA-2 the first time they are used in the manuscript.

Answer: we have defined these acronyms at the first place in the manuscript.

Question 10: Please expand your discussion of some possible high-throughput experiments that could be done with this zebrafish model of BAI that would provide clinically-relevant results.

Answer: We have expanded our discussion on possible high-throughput system based on the zebrafish BAI model in the Discussion (page 14, the 2nd paragraph).

Question 11: The authors don't state or discuss the result that there is no statistically significant difference in infection progression or macrophage infiltration at 2 dpi despite the earlier differences (in Fig 5). Is it frequently observed that early differences resolve on their own with time? What are the implications of this?

Answer: In our pervious study (Reference 31), we observed that early differences in macrophage infiltration as provoked by different types of biomaterial microspheres also resolved over time. That study however did not involve infection. Since the present study is the first with a combination of biomaterials and infection in zebrafish embryos, we do not know whether earlier differences in infection progression and macrophage infiltration due to biomaterials will generally occur.

The implications of our observations may be as follows. Firstly, the over-time resolution of early differences in *S. aureus* infection indicates that the *S. aureus* strain RN4220 was not able to "profit from" the presence of the biomaterial microspheres. This may be due to the fact that the strain has reduced virulence (*agr* negative), as we mentioned in our answer to Question 4 of Reviewer#2. Secondly, concerning the resolution of macrophage infiltration despite the presence of the PS microspheres, this suggests that PS did not strongly affect the cell responses. This is in line with the known biocompatibility of polystyrene.

Question 12: Please add information to the materials/reagents and equipment list for: chloramphenicol, bacterial incubator/shaker, zebrafish incubator, cuvettes, spectrometer, tweezers, and 48 well plates. Check spelling in the materials/reagents and equipment list.

Answer: We have added the information of the additional materials/reagents and equipment to the list and checked spelling, following the reviewer's suggestion.

Reviewer #4:

Manuscript Summary:

Overall, a good methods manuscript describing in detail a novel approach to study Staphylococcus aureus biomaterial-associated infection using larval zebrafish. The authors used fluorescent (blue)

polystyrene microspheres as the biomaterial which were co-injected with fluorescent S. aureus (mCherry) into the muscle tissue of transgenic zebrafish with labelled macrophages (GFP). The authors demonstrated that using fluorescence microscopy combined with an ImageJ plugin platform, the infection progression and leukocyte recruitment can be quantified.
The representative results of this manuscript demonstrated that indeed biomaterial may enhance the staphylococcal infection by reducing the number of recruited macrophages. All the appropriate controls were used.
Generally, the detailed protocol provided in the manuscript is accurate and should be very helpful to the zebrafish scientific community particularly for infection studies.

Major Concerns:
None

Minor Concerns:
Question 1: Although the authors mentioned that staphylococcal other strains than RN4220 can be used, RN4220 is an agr negative strain hence less virulence is expected. I believe the authors should add this information in point 1.1 of the protocol.

Answer: We thank the reviewer for this point and have added this information to point 1.1 of the Protocol.

Question 2: Approximate expected time of culturing S. aureus should be provided in point 1.1 of the protocol.

Answer: We have provided the relevant information in point 1.1 of the Protocol, following the reviewer's suggestion.

Question 3: More information about the actual injection procedure should be provided in point 4.4, such as at what angle and direction is the needle placed into the muscle.

Answer: We have provided the relevant information in point 4.4 of the Protocol, following the reviewer's suggestion.

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