**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.