

Microbiology and Molecular Genetics

Anne-Marie Krachler, Ph.D.

*Associate Professor*

September 17, 2018

**Re: Manuscript JoVE58949**

Dear Editor,

Please find enclosed our revised manuscript entitled **‘Using the protozoan *Paramecium caudatum* as a vehicle for food-borne infections in zebrafish larvae’**. We would like to thank you and the reviewers for your positive responses and really appreciate your suggestions on how to improve our work. We have updated the manuscript to reflect our response to your comments. Please find attached below a detailed breakdown of our response to the editorial and the reviewers’ comments and resulting changes to the manuscript. We hope you will now find our work suitable for publication.

Sincerely,

signature

Anne-Marie Krachler, Ph.D.

Associate Professor of Microbiology and Molecular Genetics

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**Response to Editorial comments:**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. – *We proofread the manuscript.*  
2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. –All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: falcon, Triton, FM 4-64FX, etc. – *We replaced Falcon tube with conical tube. Triton and FM 4-64 are specific reagents, which cannot be replaced by other, generic reagents.*   
3. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). *– We have edited the protocol to avoid these phrases.*4. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. *– We have amended this, except in the Discussion section where we feel these phrases are appropriate.*

Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.  
5. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:  
1.2: What is E3? Please provide its composition. What is taken from a live growing culture? It is unclear. *– We have added the composition of E3 upon its first mention in section 1.2*.  
1.3: How to measure density and what is the peak density? *– We do not actually measure the culture density, and have removed the corresponding phrasing.*  
2.1.1: What is the incubation temperature? What is used to wash the paramecia? How to count the number? Steps 4.1 and 4.2 do not exist.- *We have added the incubation temperature and amended the reference to the correct steps.*  
2.1.5: What are the selective plates and how to count CFUs? – *We have added a description of the media composition and a more detailed description of the CFU counting.*   
2.1.6: Please provide an equation if possible. – *We have now provided an equation.*  
3.1.3: Please describe how to inoculate bacterial growth media with infectious strain of bacteria. Please provide composition of bacterial growth media. – *We have added the medium composition and procedure.*   
3.1.6: Step 4.1.5 does not exist. Should it be 3.1.5? *Yes, corrected.*   
3.1.8.3: What is used to wash? Please specify the wash step repeated here. – *We added that the wash is performed with 1xE3.*   
3.2.3: What is used to remove the supernatant? – *Added that a serological pipette is used.*3.2.9: What is the incubation temperature? – *Added the incubation temperature.*3.3.1: Steps 4.2.10 and 4.2.6 mentioned in this step do not exist. Please revise. – *Amended.*  
3.3.3: Step 4.3.2 does not exist. Please revise.- *Amended to 3.3.2.*  
6. 2.2 and sub-steps: Can these steps be moved after step 3.3.4. As currently organized, the readers/viewers have to refer back and forth in order to complete the protocol.- *We have moved this section to the end of the protocol.*   
7. 2.3.1 and 2.3.2: Please break up into sub-steps.- *We broke it up into sub-steps.*  
8. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step. – *We have combined steps wherever we thought possible.*   
9. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.  
10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia. Please note that calculation steps are not appropriate for filming.  
11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.  
12. Figure 2: Please state the unit of time in panel A in the figure legend. *– Included in figure legend.*  
13. Discussion: Please discuss any limitations of the technique and the significance with respect to existing methods. *– We have included some more discussion of limitations and comparison with existing techniques.*   
14. References: Please do not abbreviate journal titles.  
  
**Reviewers' comments:**  
  
**Reviewer #1:**  
Manuscript Summary:  
good, detailed procedure to produce a useful experimental tool. Only minor concerns that can be addressed in text and possibly with addition of already existing data  
  
Minor Concerns:  
Line 249 reference to 4.1.5 should be 3.1.5 – *Thank you, this has been corrected.*  
274 insight into gravitational tolerance of paramecium would be nice. Does harder for shorter kill the paramecium or can they tolerate a shorter spin at higher g? – *We use the minimal force required to pellet them efficiently, and see no benefit in increasing the spin force.*   
277 recommend pipette or decanting to rapidly remove supernatant without disturbing motile pellet? *– We added to add a serological pipette for this step.*   
296 I assume a standard 4% PFA? PFA is missing from materials section – *We have specified the formaldehyde concentration and added the reagent to the materials list.*  
320 why is it necessary for the embryos to be knocked out prior to transfer*? – Anesthetizing the embryos facilitates fast and efficient transfer and minimizes distress and risk of causing damage to the animals.*   
324 please add necessity of lighting to achieve high/reproducible prey rates. Is lab bench lighting sufficient or is more direct light required? – *We have added the note: ‘Incubation should be carried out in a diurnal incubator under day-light conditions, to ensure optimal lightning conditions for preying.’*  
333 add homogenisation and plating steps and indicative results – *The halflife experiment does not as such contain a homogenization step. The plating step is described in step 2.1.5, and representative results in Fig. 1.***Reviewer #2:**  
Manuscript Summary:  
ln this manuscript, Flores and colleagues describe using paramecia as a delivery system for pathogenic bacteria into the intestines of larval zebrafish. Zebrafish is increasingly being used as a model for investigating gnotobiology and bacterial pathogenesis, so this approach is timely and should be very useful to the research community. However, as described below, there are issues that need to be resolved before this manuscript can be published and used as the basis for a video.  
  
Major Concerns:  
1) At the beginning of the protocol, the experiments are described as being in accordance with the Animal Scientific Procedures Act 1986 which is from the UK. Since the work is covered by a University of Texas Health Science Center Institutional Animal Welfare Committee protocol number, the authors should change this to the Guide for the Care and Use of Laboratory Animals, or something else specific to the US. *– The section has been amended accordingly.*  
2) A list of abbreviations and solutions would be useful. – *we have added the composition of E3, and formaldehyde, which are the only relevant solutions.*   
  
3) In step 2.1.2, are the samples from different time points added to the same 1.5mL tube? Or is each sample added to a different tube? It doesn't make sense to me why all of the samples would be added together. But reading the protocol it isn't clear that they aren't. Assuming there are different tubes for each time point, then 2.1.3 should say tubes plural, rather than tube singular, which reinforces the idea that all the samples go into a single tube*.- They all go into separate tubes, and we have edited the section to improve clarity.*  
  
4) In step 2.2.2 and lines 352-359 the authors define the number of prey capture events as the number of strikes. They acknowledge, in the discussion, that the number of strikes and the number of prey captures aren't necessarily the same. I think it would be useful to include that information here, or at least include a reference to the discussion. – *We have added references to the Discussion in step 2.2.2. and in the representative results section, as suggested.*   
  
5) It's not at all clear what is shown in Figure 2. The authors should have pictures that include both a larva and the fluorescent bacteria. And why is the entire larva, including the eyes, fluorescing in red? – *As explained in the legend, Figure 2 shows a larva preying on paramecia that carry internalized bacteria (small round objects in the frames). Single bacteria would be too small to visualize at this magnification. To visualize the larva movements, we have to use both the red fluorescent channel, as well as a minimal amount of bright field backlighting. The latter leads to the red appearance of the larvae, which is the only way to track both larva and paramecia for these videos.*

6) How do the authors know that the bacteria in Figure 3 are alive? *Dead bacteria, when visualized under the microscope, rapidly lose membrane integrity and thus, fail to contain fluorescence.*   
  
7) Most of the fluorescence in Figure 3 is in intestinal epithelial cells, not in bacteria. It would be useful to include a blow up that shows both the autofluorescent intestinal epithelial cells and the fluorescent bacteria. *– We have added an uninfected control fish to Figure 3, which highlights that there is little to no autofluorescence in the red channel coming from the epithelial cells. The reason the epithelium appears red is because the bacteria are tightly associated with the mucosae and, to some extent, invade the epithelium. To highlight this, we have added a series of higher magnification images (Fig. 3D), which show bacterial localization in respect to the intestinal epithelium.*

8) The equations in 3.3.1 have several errors. First, there is no step 4.2.10. Second, I don't see how paramecia concentration/dilution factor can equal the dilution factor. Third, there is no step 4.2.6. – *We have amended the formula, to reflect both the correct step numbers as well as the correct algebra.*   
  
9) What is the dosing experiment of step 3.3 that is referred to in 3.3.2? – *We have edited the text to read ‘ The concentration of paramecia can be adjusted based on the desired bacterial dosage, which is subject to optimization.’*  
  
10) 3.3.3 would be easier to follow if broken up into two steps - first anesthetize the larvae and place them in a well with fresh E3 - and second add the paramecia. Alternatively, first prepare the well with fresh E3 and paramecia - and second add anesthetized larvae. It also needs to be made clear whether the larvae are still anesthetized when they are in the well with the paramecia. I wouldn't think so, as they wouldn't be able to hunt, but this isn't clear from the manuscsript. If the larvae are not anesthetized when they are in the well, why are they anesthetized first? Is this just for handling purposes? And when is the anesthesia removed? This should all be clarified. – *We have edited this step for clarity. The larvae are anaesthetized for ease of handling and to reduce the risk of damage and distress. The target well does not contain tricaine, and care has to be taken to transfer the larvae with a minimal amount of liquid to ensure they recover from anesthesia once they reach the recipient well.*   
  
11) In 3.3.5 are the fish transferred from well to well for the five washes? Or is the tricaine E3 sucked out of the well? Does this matter? Does it matter whether the larvae are anesthetized? – *Yes, and each well has E3 plus tricaine. This minimizes chances to do damage and cause distress to the animal, which we have now added to the text.*  
  
12) More information should be given about the embedding step in 3.3.6. – *We have added more detail regarding the embedding step.*   
  
13) Line 222-223 refer to video 1 which was not made available to review. – *We have added the video to the uploaded files.*   
  
Minor Concerns:  
1) Unclear why some of the protocol has yellow highlighting*.- This is a requirement of the journal for video purposes.*  
2) What are the units of preying rate? *[s-1]*  
  
3) In 3.1.3, or elsewhere, it would be prudent to say something about appropriate PPE to wear while working with infectious bacteria. – *We have added a note to that effect to the inoculation step.*  
4) 3.1.6 refers to 4.1.5. But there is no 4.1.5. – *Amended to 3.1.5.*  
  
5) There is an extra with in 3.1.8.2. – *Thank you, has been removed.*  
6) More detail on the washing would be useful in 3.1.8.3. – *We have specified number, volume, and media used for washes, as well as centrifugation speed to be used.*   
  
7) 3.1.9 has an extra the. – *Thanks, this has been removed.*  
  
8) Why are two flasks used in 3.1.9 and then the contents combined in 3.2.1? – *We use 2 T-25 flasks containing 10 ml each, since this guarantees optimal aeration of the cultures.*   
  
9) In 3.2.6 is the remaining E3 what is left in the tube after removing as much as possible in the previous step? *We edited this and the prior steps to enhance clarity.*   
  
10) What is the final concentration of tricaine used for anesthesia? *For transfer steps, we use 0.1 mg/L of tricaine, and for embedding and long-term procedures 160 mg/L tricaine, as noted in the manuscript.*

**Reviewer #3:**  
  
Manuscript Summary:  
This protocol describes the use of the protozoan Paramecium caudatum as a vehicle for food-borne infection in zebrafish larvae.  
  
Major Concerns:  
1. Is there a stable phase of bacteria dose in the paramecia after co-incubation? – *There might be a short (few minute-long) plateau prior to acidification which might be considered a stable phase. However, since this timeframe is very short in comparison to co-incubation and subsequent gut transit time, we found it more practical to consider bacterial half-life using the formulas presented in the manuscript to work out the dose of viable bacteria at any given point during incubation.*

2. Is it considered that the bacteria in the feces could be prey again? *– Over the timeframe of prey exposure (2hrs – gut passage time is more than 1 hr), and considering the relative concentration of bacteria in paramecium vs in feces, this constitutes a negligible contribution.* We have added this consideration to the discussion.  
3. Line 304. Counting the dead paramecia. In co-incubation of Paramecia and zebrafish larvae protocol, whether the dead paramecia should been removed? –*The paramecia will be dead because of the formaldehyde fixation at this point, but this number will reflect the concentration of live paramecia for the co-incubation experiment. We have determined that co-incubation with bacteria does not result in significant death of paramecia, but rather in proliferation, so this can be disregarded and no removal is necessary. We have added a note to explain this.*

Minor Concerns:  
Explain the E3 medium.- *We have added a description of contents upon first mention.*