Dear Editor, and Reviewers 1, 2,

The authors of the submission entitled ‘High-throughput measurement of gut transit time using larval zebrafish’ would like to thank the editor and reviewers for their helpful critique and suggestions for our manuscript. We have striven to clarify and make changes to help improve our manuscript accordingly. Please see descriptions of those steps below, as well as responses to questions. As there were many changes, for clarity we will provide a manuscript version with changes accepted; the version of the manuscript with changes ‘tracked’ will be provided as a supplementary document.

Thank you again for your help, and consideration to publish our work.

Steven Cassar

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

The manuscript was proofread and minor changes made to typographical errors.

• Textual Overlap: Significant portions show significant overlap with previously published work. Please re-write the text indicated in red in the attached document to avoid this overlap.

The text was re-written in the indicated portions to avoid textual overlap.

• Protocol Highlight: The animations referenced in 6.4.1 can likely be shown in the introduction. It will be great if you provide a brief description accompanying this animation in a separate supplementary file.

A suggested script was written along with guidelines as to the words to be said with the timing of the animation; this is provided as supplement material.

• Results: Please mention the statistical tests performed and report sample sizes. Also mention the significance level assumed.

The statistical analysis and significance level are now included in the description of the results.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail: modifications and troubleshooting, limitations of the technique, significance with respect to existing methods, future applications and critical steps within the protocol.

These points have been added to the Discussion, including critical steps, comparison to other techniques, technical challenges and suggestions for further optimization, and early troubleshooting efforts during the assay’s development.

• Figures:

1) Please add scale bars to 1A. The new Figure 1A now has a scale bar.

2) Please define the error bars in fig 2 and 3. These error bars are now defined in the legends.

• References:Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows (please do not abbreviate journal titles): (For 6 authors or less list all authors. For more than 6 authors, list only the first author then et al.): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, doi:DOI (YEAR).]

The references have been changed to comply with the format.

• Commercial Language:JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Example of commercial sounding language in your manuscript is fluospheres.

1) Please use MS Word’s find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.

2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

Fluospheres has been removed and replaced with fluorescent label; any other reference to trademarked items were removed.

• Table of Materials: Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as drugs, fish strain, etc.

The table of materials has been accordingly changed to include additional items and catalogue numbers as requested.

• Please define all abbreviations at first use.

Abbreviations are now defined at first use.

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

Standard abbreviations and symbols are now used, and a space is between numbers and unit.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Republishing permission is now provided in a separate document as supplementary material; the original publication is now cited in the figure legends.

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Comments from Peer-Reviewers:

Reviewer #1:

The manuscript submitted by Cassar and coauthors is useful, accurate, and in this reviewers opinion it will help the reader to implement the assay. It is a good addition to the literature. It will compliment a JOVE video reporting the assay. I hope it is open-access!

I believe it will be open-access.

I did not mention minor edits because the JOVE editors will do this. The bullets below are comments from manuscript review. All are simple, and minor.

One additional overall comment is that tis manuscript is written from a toxicology perspective. I agree that this is the most common use for this assay. However, what about drug discovery? Certainly we do not have sufficient effective prokinetic agents available to treat dysmotility? Second, using the zebrafish to delete or add specific genes and/or cell populations and using this assay to test effectiveness would be a useful and feasible application. For example, deficits in enteric neurons are well described in patients A model with 60% of the ENS 'deleted' would be an excellent starting point for compound development.

The Discussion now contains text describing some ‘discovery’ applications for which the assay may be useful, including those mentioned by this Reviewer; thank you.

The title and abstract are appropriate.

The materials and equipment needed listed are sufficient.

The steps listed in the procedure are sufficient.

All steps are clearly explained.

Appropriate controls are suggested and critical steps are highlighted.

Some anticipated results may be un-expected (see comments below).

References are fine.

NOTES

\*Abstract: should spectrophotometer read 'plate reader'?

Where spectrophotometer was before, it now reads ‘plate spectrophotometer’ for clarity.

\*'the average is plotted'. Isn't variance an important measure as well?

Variation around the mean is now mentioned, in the text, as being plotted and considered in statistical analysis; also, the error bars are explained in the figure legends.

\*HOW MUCH compound is used? Better the 'very little'.

This phrase was removed to avoid textual overlap.

\*How does this compare to cell culture?

Cell culture is mentioned for comparison in the Discussion, now.

Short Abstract: The goal is fine, if that is what you really mean. But can't the assay be used for other things? For example, to examine the role of specific genes on GI transit time? If that is the case, then then short abstract might be re-written. One suggestion:

The goal of this protocol is the measure the transit time of a non-digestible fluorescent marker through the gastrointestinal tract of larval zebrafish in a high throughput fashion.

The short abstract was changed based on this good suggestion.

Long Abstract:

\*I am unsure if you mean to interchange 'compound' with 'drug'.

Now, compound is used only when referring to test article, and drug is used in reference to marketed drugs, or drug discovery.

\*Is fluorescent food identical to food with a fluorescent marker?

Now ‘fluorescent food’ is defined as referring to food containing a fluorescent label, early on in the Methods.

Protocol:

\*Egg water is given as 60µg/mL = 0.06g/L = 60 mg/L. I think that is commonly used for adults (Zebrafish Book). But some prefer to use embryo medium for larvae. And egg water is different still, but is according to your recipe.

Now egg water is referred to as embryo medium = 60 mg/L.

\*Transfer larvae: do you anesthatize larvae when you do this? Isn't it a challenge to grab individual larvae with a P1000? Set to 100µl?

\*How sensitive is the plate reading to movement? What is the plate reader is not in the fishrom or near the incubator, for example?

\*Many plate readers have heaters. Can those be used?

The 3 points/questions above are addressed, now, in the Discussion.

\*Is evaporation an issue?

\*Is it tricky to set the range for the plate reader?

\*When collecting light is there a certain time, or is this pre-set in plate readers?

The 3 questions above are not addressed in the new manuscript; plate readers vary, but usually are easy to program and are preset with default emission collection timing – ours worked for us. The question on evaporation was not interrogated by our work and we don’t believe that it is an issue; the manufactured lit was kept on the microplates to reduce evaportation.

\*Line 280: Is it possible that the atropine signal is lower compared to vehicle control because the control larvae have something in the GI tract that fluoresces? This substance would therefore progress through the GI tract faster compared to atropine-treated animals. Also, was it compared, possibly, to a no-larvae control, for example? It would be surprising if that value changes over time. But if it does then correcting or that drift would be appropriate.

The possibility that something in the GI tract fluoresces is now suggested as a possibility in the Discussion. Our data indicate no signal (0) from wells containing no larvae. We did not examine the transited material from larvae fed un-labeled food, so we don’t have any data on endogenous fluorescence.

\*It is also written that agents that accelerate transit time result in HIGHER readings when compared to vehicle. Isn't that predicted?

Higher readings are predicted early during the course of gut evacuation, however higher final readings are not predicted. This is now clarified in the discussion.

\*The animation is a nice addition. But- doesn't fecal accumulation level out? It might be a little better if the animation showed that.

Although a good suggestion, we did not change this as the animation only provides the basic idea of how the assay works.

Reviewer #2:

Manuscript Summary:

High-throughput measurement of gut transit time using larval zebrafish

The text portion of this JOVE submission is satisfactory for the most part. A list of abbreviations would help the reader and should be added.

A list of Abbreviations has been added – after Keywords.

Major Concerns:

none

Minor Concerns:

Line 94: when removing dead/unfertilized eggs/embryos, it seems additional embryos should be added, especially if a significant number are removed, so all dishes have the same number thus equal access to the 2ug of food.

This is now clarified in the methods.

Since feeding is begun before 7 days with unlabeled food, the authors should indicate if any autofluorescence is present in the gut contents prior to feeding fluorophore spheres.

Reviewer 1 asked a similar question; we did not examine the transited material from larvae fed un-labeled food, so we don’t have any data on endogenous fluorescence.