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TITLE:

High-Throughput Measurement of Gut Transit Time Using Larval Zebrafish

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KEYWORDS:

Drug, Gastrointestinal, Methods, Safety, Zebrafish, Alternative

SHORT ABSTRACT:

The goal of this protocol is to measure the transit time of fluorescently labeled food through the gut of larval zebrafish in a high throughput fashion.

LONG ABSTRACT:

Zebrafish are used as alternative model organisms for drug safety testing. The gastrointestinal (GI) tract of zebrafish has genetic, neuronal, and pharmacological similarities to that of the mammals. GI intolerance during clinical testing of drug candidates is common and may pose a serious threat to human health. Testing for GI toxicity in preclinical mammalian models can be expensive in terms of time, test compound, and labor. The high-throughput method presented here may be used to predict GI safety issues. Compared to mammalian models, this method allows for more expedient assessment of test compound effects on GI transit while using low quantities of compound. In this method, larval zebrafish (7 days post fertilization) are fed food containing a fluorescent label. After feeding, each larval fish is placed into a well of a 96-conical-bottom-well plate and dosed with test compound (dissolved in water) or the vehicle. As gut transit occurs, fecal matter accumulates on the bottom of the wells, and the rate at which this happens is monitored by measuring fluorescence from the bottom of the well repeatedly over time using a plate spectrophotometer. The fluorescence from larvae in a given treatment group are averaged and these values are graphed along with standard error, for each measurement time, yielding a curve representing average transit of food over time. Effects on gut transit time are identified by comparing the area under the curve for each treatment group to that of the vehicle-treated group. This method detected changes in zebrafish GI transit time induced by

drugs with known clinical GI effects; it can be employed to interrogate dozens of treatments for GI effects per day. As such, safer compounds can be quickly prioritized for mammalian testing, which expedites discovery and proffers 3Rs advancement.

INTRODUCTION:

Zebrafish (*Danio rerio*) are used to model vertebrate biology and predict drug toxicity and/or efficacy; new applications in these fields emerge each year. The advantages of zebrafish over mammalian models include their fecundity, small size, and transparency through organogenesis. Zebrafish are used to predict drug candidate acute toxicity, as well as to assess compound impact on organ function, *e.g.,* cardiac, ocular, gastrointestinal (GI)[1, 2]. Zebrafish development and physiology are similar to those of mammals in many ways[3] and 80% of genes that are associated with human disease have a zebrafish homolog[4].

The GI tract of zebrafish has similar physiology to the mammalian GI tract but has a simpler architecture[5]. Zebrafish have no stomach; the anterior intestinal bulb acts as a food depot. Gene expression in zebrafish intestine has many similarities to that of the mammal[5]. Like mammals, the enteric nervous system of zebrafish controls gut motility, and intestinal innervation mirrors that of other vertebrates[6, 7]. Based on these similarities, functional disorders of the human intestine have been studied in zebrafish, using methods derived from mammalian models[8].

GI intolerance during clinical testing of drug candidates is common and may pose a serious threat to human health. A review of programs in a major pharmaceutical company during 2005–2010 revealed GI safety as the principal cause for 9% of clinical study terminations[9]. Testing for GI toxicity in preclinical mammalian models can be expensive in terms of time, compound, and labor. A predictive high-throughput assay for GI transit can provide flexibility to compound toxicity testing, and deliver 3Rs impact. A novel method offering such an assay is presented by the protocol described herein. This high-throughput assay could be employed early in drug development to prioritize candidates, and contribute to the reduction of GI safety testing in larger species.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of AbbVie. Abbvie operates under the National Institutes of Health Guide for Care and Use of Laboratory Animals in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). No animal health concerns were observed in these studies.

1 Breed Adult Zebrafish and Collect Embryos

1.1 House and breed adult zebrafish using general husbandry and breeding practices. For example, see Westerfield[10].

1.2 Prepare embryo medium by dissolving dehydrated sea salt (see **Table of Materials**) in deionized water at a concentration of 60 mg/L.

1.3 Collect fertilized eggs from the adult breeding chamber, rinse well with embryo medium and house embryos in about 50 mL of embryo medium in 10 cm Petri dishes (50 embryos per dish) at 28 ± 1 °C on a 14:10 h light:dark cycle.

1.4 Remove non-surviving embryos after 24 h and supplement with surviving embryos so that each dish contains 50 embryos per dish.

2 Train Larvae to Feed Using Non-Dyed Food

2.1 On the 4th day post-fertilization (4 dpf), feed the larvae in each Petri dish 2 mg of powdered larval fish food (see **Table of Materials**) by sprinkling the food on top of the water.

2.2 Allow the larvae 3–4 h to feed and then transfer them to a clean (no food) Petri dish containing about 50 mL fresh embryo medium.

2.2.1 To aid in transferring as little food as possible, rinse each larva in a Petri dish containing about 50 mL fresh embryo medium before the final transfer to the new dish.

2.3 Repeat feeding and rinsing of the larvae on 5 dpf and 6 dpf.

3 Prepare Fluorescent Food on the 6 dpf and Feed to Larvae on the 7 dpf.

3.1 Prepare food containing a fluorescent label (hereafter referred to as fluorescent food, according to methods of Field *et al.*[3]). Briefly, mix 300 µL of fluorescent label (see **Table of Materials**), 100 µL of deionized water, and 200 mg of powdered larval food on a 10 cm watch glass.

3.2 Spread the resulting paste into a thin layer on the watch glass. Allow the paste to dry at room temperature in the dark for >8 h.

3.3 Scrape the dried mixture off the watch glass, crush to powder, and store at room temperature in the dark. The fluorescent food is now ready to be fed to the larvae.

3.4 On the 7 dpf, feed the fluorescent food to larvae in the same manner as done for previous feedings, that is provide 2 mg fluorescent food per dish (see steps 2.1–2.2.1).

Note: Ensure that the food is finely ground to a powder. Rubbing fluorescent food that is wrapped in weighing paper between thumb and forefinger is a useful method for ensuring finely ground food.

4 Prepare Concentrated Dosing Solutions of Test Compounds

4.1 Dissolve each test compound in embryo medium to a concentration that is 2× the target dose. If testing of a dose range is desired, prepare multiple concentrated dosing solutions of the appropriate concentrations for the desired doses.

4.2 Prepare enough of each concentrated dosing solution so that 24 larvae can be treated. Each larva will require 100 µL dosing solution (the final volume per well is 200 µL), so at the very least, 2.4 mL dose solution is needed for each treatment group; 2.5 mL would be an appropriate volume.

4.3 If a solvent (*i.e.,* dimethyl sulfoxide) was used for initial solubilizing of test compound, prepare the appropriate vehicle control dose (*i.e.,* same amount of solvent as the compound treatment but without the compound). And, as was done for each compound-treated group, prepare enough vehicle solution to treat 24 larvae.

5 Transfer Larvae to a 96-well plate and Apply Treatments

5.1 After larvae have been allowed to feed on fluorescent food for 2 h, use a transfer pipette to move them to a rinsing dish, as was done after feeding on prior days.

5.2 After each larva is rinsed, withdraw it along with 100 µL embryo medium, and move it into a well of a 96-well polystyrene conical-bottom multi-well plate (see **Table of Materials**), dispensing the full 100 µL embryo medium into the well with the larva.

5.3 Once the required numbers of larvae have been transferred to the 96-well-plate, add 100 µL of the 2× concentrated dosing solutions to each well.

Note: The use of a 12-channel multichannel pipette facilitates rapid dosing of the larvae (12-at-a-time). To avoid accidentally adding the wrong treatment, keep close track of which larvae have been dosed with which treatment and be sure to change pipette tips between treatments.

6 Measure Initial and Subsequent Fluorescence from Each Well

6.1 After adding the dose solutions, place the 96-well-plate into a plate spectrophotometer capable of exciting and detecting emissions from the fluorescent label.

Note: For the yellow-green label used (see the **Table of Materials**), the appropriate wavelengths of light are exciting at 505 nm and detecting at 515 nm.

6.2 Read the fluorescence of the 96-well-plate from the bottom 5 times in immediate succession without shaking the plate. Use the minimum value of the 5 readings from each well as the initial fluorescence from the well.

Note: The reason that 5 readings are taken whenever the plate is read is that larvae sometimes swim in the path of the excitation light and the food in their gut emits a very large signal which

is unrepresentative of the released feces. Taking the minimum of the 5 reads can help to avoid using artificially high measurements from un-transited food.

6.3 Read the fluorescence of the 96-well-plate (as was done for the initial reading) every 20 min for the first 2 h post-dosing, every 30 min for hours 3 and 4 post dosing, and then once every hour for hours 5, 6, 7, and 8 post-dosing.

6.3.1 Take caution to not disturb (by shaking the 96-well-plate) settled fecal matter between reads and incubate the larvae at 28 ± 1 °C between reads.

6.4 Incubate the larvae over night at 28 ± 1 °C and read the fluorescence from the 96-well-plate the following morning around the same time that the larvae were dosed the day before. Use this measurement as the 24 h post-dose fluorescence.

7 Analyze the Data

Note: Here we calculate accumulation per well and group averages for each time point.

7.1 To calculate accumulation per well, using only minimum values from each of the 5 reads, subtract the initial value from each time point's value. Perform this calculation for the initial value as well (this results in an initial accumulation of zero for each well).

7.1.1 If the accumulation in a well at the 24 h time point is less than 150 relative fluorescent units, exclude that well from further analysis; these low values are most likely due to low or no intake of the fluorescent food by the larvae during feeding, and thus those larvae are not good subjects for measuring transit time.

7.2 For each time point, calculate the average accumulation for wells containing co-treated larvae, as well as standard error of those averages.

7.3 Plot the average accumulation on the Y-axis *versus* time on the X-axis for each treatment group and compare the areas under those curves (AUCs).

Note: Treatments that significantly slow or accelerate transit time will have AUCs significantly smaller or larger, respectively, than the vehicle-treated group.

REPRESENTATIVE RESULTS:

This method, which uses plate-based spectrophotometry to assess GI transit, can be used as a high-throughput replacement of fluorescent microscopy, which is a lower throughput method for assessing the same function (**Figure 1**). To generate the data in **Figure 1**, identically treated fish were analyzed for GI transit using either fluorescence microscopy (representative images shown) or spectrophotometry at 4 time points, 0, 4, 8, and 24 h post-dosing; comparison of the data from those experiments gave highly correlated results (linear regression of data $r^2 = 0.95$).

The linear regression has a negative slope because microscopy measures the retained fluorescence signal and spectrophotometry measures the transited signal.

The effects of compounds of disparate mechanisms, with well-established GI activity in humans, can be detected in zebrafish using the spectrophotometry assay (**Figure 2**). Compared to vehicle-treated controls, atropine (4.2 µM) and amitriptyline (5 µM) slowed GI transit, while tegaserod (3.3 µM) and metoclopramide (33 µM) accelerated transit time. Erythromycin (14 µM), expected to accelerate transit time had no effect as measured by this method. Treatment group sizes were 24 before removing data from larvae with no or very low signal. The AUC for the average signal per time point was compared between compound-treated and vehicle-treated groups using Tukey's Honestly Significant Difference for type-1-error control. Effects were considered significant only when $p \leq 0.05$. The concentrations used for the above treatments were the maximum tolerated doses, determined in a prior experiment and defined as the highest dose with no observable adverse effect by gross observation.

The spectrophotometry assay can measure dose-dependent effects of compounds. **Figure 3** provides data demonstrating that atropine slows GI transit dose-dependently in zebrafish larvae. The lowest dose of atropine tested, 0.042 µM, had no significant effect, while the two higher concentrations each had significant impact, 0.42 µM having less of an effect than 4.2 µM.

A new assay can be assessed by testing positive and negative controls, that is, compounds known to be active and inactive respectively in the target system (in this case the target system is the mammalian GI transit). For the spectrophotometry assay, 18 active (positive) controls and 6 inactive (negative) controls were tested. Based on these experiments, the spectrophotometry assay has high positive predictive value (90.9%), but low sensitivity (55.6%) and negative predictive value (38.5%). These values are derived from the data presented in **Table 1**. They reflect, in practice, that if the zebrafish transit time is impacted by a treatment, mammalian transit is likely to be impacted. However, if there is no effect on zebrafish transit time, this is not predictive of mammalian effect.

FIGURE AND TABLE LEGENDS:

Figure 1. **Fluorescent food transit is detected as a loss of signal from microscopic imaging and a corresponding gain in signal by plate-based spectrophotometry. A**) Representative microscopic images from analysis of atropine (4.2 µM) effect on GI transit time. **B**) Average signal quantified from microscopic images is highly (negatively) correlated with signal from voided fecal matter (spectrophotometry) from identically treated fish. Data from atropine-treated fish are in red. This figure copied with permission from Cassar *et al.* [11].

Figure 2. Analyzing fluorescent signal accumulation over time from a multi-well plate allows identification of treatments that change GI transit rate. Asterisk (*) indicates significantly different AUC than vehicle-treated fish. Error bars represent the standard error of the mean signal for larvae in the treatment group per time point. This figure has been reused with permission from Cassar *et al.* [11].

Figure 3. **Atropine dose-dependently slows zebrafish gut transit time as reflected by fluorescent spectrophotometry of fecal accumulation over time.** Asterisk (*) indicates significantly different AUC than vehicle-treated fish. Error bars represent the standard error of the mean signal for larvae in the treatment group per time point. This figure is reused with permission from Cassar *et al.* [11].

Table 1. **GI activity of 24 compounds in mammals and fish.** This table is reused with permission from Cassar *et al.* [11].

DISCUSSION:

The novel spectrophotometry method for measuring zebrafish larvae GI transit time, presented here, is an efficient assay that can predict treatment effects on mammalian GI-function. Although the assay has low sensitivity, it has high positive predictivity, which is acceptable for first tier assays employed for paring down the number of candidate treatments based on toxicity[12]. This method is easier to execute, has higher throughput, and uses less animal handling steps than fluorescent microscopy.

There are technical challenges inherent in this method. Catching individual larvae after feeding the fluorescent food and transferring them into individual wells is challenging at first. However, with practice, a skilled technician can fill a 96-well plate in less than 15 min. If, at a given time point, the plate is accidentally shaken and the fecal matter unsettled from the bottom of the wells before reading, the accumulation will appear to decrease. This can be avoided by moving the plate(s) carefully, without shaking. In our experience, normal movement, including that of the plate spectrophotometer motorized drawer, did not disturb the assay. Plate readers equipped with a heater (*i.e.*, the plate does not have to be returned to incubator between measurements), and located near the assay lab bench could optimize for lower chance of disturbance, but this was not necessary in our experience.

Early attempts at the method did not include feeding on the days before the assay. Without those 'practice' feedings, lower numbers of larvae consumed sufficient fluorescent food during the time allowed before treatment application. In those attempts, variation within treatment groups was greater, and more data was unusable due to low/no signal accumulation over time. Even with practice feeding, some larvae do not consume sufficient amounts of the fluorescent food, excluding data from those larvae decreases variation within groups and increases the ability to identify treatment effects. Starting with larger group sizes (*i.e.,* 24 *versus* 12) allows for sufficient numbers of larvae contributing useful data to the analysis.

The fluorescent microscopy reveals that food transit was nearly complete by 24 h in atropine-treated larvae (not shown), however the microplate data from the final time point reflect lower final signal in the atropine group, compared to vehicle group. Conversely, higher final microplate fluorescence was associated with treatments that accelerated transit time, even though the vehicle-treated larvae have apparently voided their GI tract before the final time point. Based on the random assignment to treatment of larvae fed fluorescent food, we assume equivalent

consumption of the fluorescent food, on average, among treatment groups. Given this, and based on the patterns described above, fluorescence from fecal matter declines with time spent in the larval GI tract or faster transit adds fluorescence to the feces somehow. The actual cause is unknown and has not been interrogated, however the assay still is capable of measuring and comparing transit time among groups.

The employment of this method in detecting toxic GI effects from small molecule compounds is one application. Other possible applications include disease modeling (*e.g.*, irritable bowel syndrome) and novel therapy discovery for such diseases, or for discovering pro-kinetic compounds. Furthermore, coupled with transgenic models, this method could be used to interrogate the role of genes in normal GI transit, as well as transit disorders, including enteric neuron deficiency. The zebrafish larva offers a whole organism platform on a scale that approximates that of cell culturing, but since there are multiple tissues and myriad cell types functioning together in the zebrafish, systems biology questions can be asked and answered using this model.

With advancements in technology, and new applications thereof, efficiency in the conduct of zebrafish toxicity tests will continue to improve. Larval zebrafish handling methods and assays are continuing to improve in terms of higher throughput[13, 14]. The novel method presented here is one example of an improvement that may make zebrafish studies more impactful.

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Simon Cassar of carpetbones.com designed and created the animated figure used to demonstrate the in-life procedure of the GI transit assay.

DISCLOSURES:
The design, study conduct, and financial support for this research were provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the manuscript. S. Cassar, X. Huang, and T. Cole are employees of AbbVie and have no additional conflicts of interest to disclose.

REFERENCES:
1. Berghmans, S. *et al.* Zebrafish based assays for the assessment of cardiac, visual, and gut function — Potential safety screens for early drug discovery. *Journal of Pharmacological and Toxicological Methods.* **58** (1), 59–68, doi:10.1016/j.vascn.2008.05.130 (2008).

2. Field, H. A., Kelley, K.A., Martell, L., Goldstein, A.M., Serluca, F.C. Analysis of gastrointestinal physiology using a novel intestinal transit assay in zebrafish. *Neurogastroenterology and Motility.* **21**, 304–312, doi:10.1111/j.1365-2982.2008.01234.x (2009).

3. Kanungo, J., Cuevas, E., Ali, S., Paule, M.G. Zebrafish model in drug safety assessment. *Current Pharmaceutical Design.* **20** (34), 5416–5429, PMID:24502596 (2014).

4. Howe *et al.* The zebrafish reference genome sequence and its relationship to the human genome. *Nature.* **496**, 498–503, doi:10.1038/nature12111 (2013).

5. Shepherd, I., Eisen, J. Development of the zebrafish enteric nervous system. *Methods in Cell Biology*. **101**, 143–160, doi:10.1016/B978-0-12-387036-0.00006-2 (2011).

6. Holmberg, A., Olsson, C., Holmgren, S. The effects of endogenous and exogenous nitric oxide on gut motility in zebrafish *Danio rerio* embryos and larvae. *The Journal of Experimental Biology.* **209**, 2472–2479, doi:10.1242/jeb.02272 (2006).

7. Olsson, C., Holmber, A., Holmgren, S. Development of enteric and vagal innervation of the zebrafish (*Danio rerio*) gut. *The Journal of Comparative Neurology.* **508**, 756–770, doi:10.1002/cne.21705 (2008).

8. Fleming, A., Jankowski, J., Goldsmith, P. *In vivo* analysis of gut function and disease changes in a zebrafish larvae model of inflammatory bowel disease: A feasibility study. *Inflammatory Bowel Disease.* **16** (7), 1162–1172, doi:10.1002/ibd.21200 (2010).

9. Cook, D. *et al.* Lessons learned from the fate of AstraZeneca's drug pipeline: A five-dimensional framework. *Nature Reviews Drug Discovery.* **13** (6), 419-431, doi:10.1038/nrd4309 (2014).

10. Westerfield, M. *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio Rerio)*. University of Oregon Press. Eugene, OR, USA. (2000).

11. Cassar, S., Huang, X., Cole, T. A high-throughput method for predicting drug effects on gut transit time using larval zebrafish. *Journal of Pharmacolical and Toxicological Methods*. **76**, 72-75, doi:10.1016/j.vascn.2015.08.156 (2015).

12. McKim, J.M., Jr. Building a tiered approach to *in vitro* predictive toxicity screening: A focus on assays with *in vivo* relevance. *Combinatorial Chemistry and High Throughput Screening.* **13** (2), 188–206, PMID:20053163 (2010).

13. Bruni, G., Lakhani, P., Kokel, D. Discovering novel neuroactive drugs through high-throughput behavior-based chemical screening in the zebrafish. *Frontiers in Pharmacology*. **5**, 153, doi:10.3389/fphar.2014.00153 (2014).

14. Mandrell, D. *et al.* Automated zebrafish chorion removal and single embryo placement: optimizing throughput of zebrafish developmental toxicity screens. *Journal of Laboratory Automation*. **17**, 66–74, doi:10.1177/2211068211432197 (2012).

Figure 1

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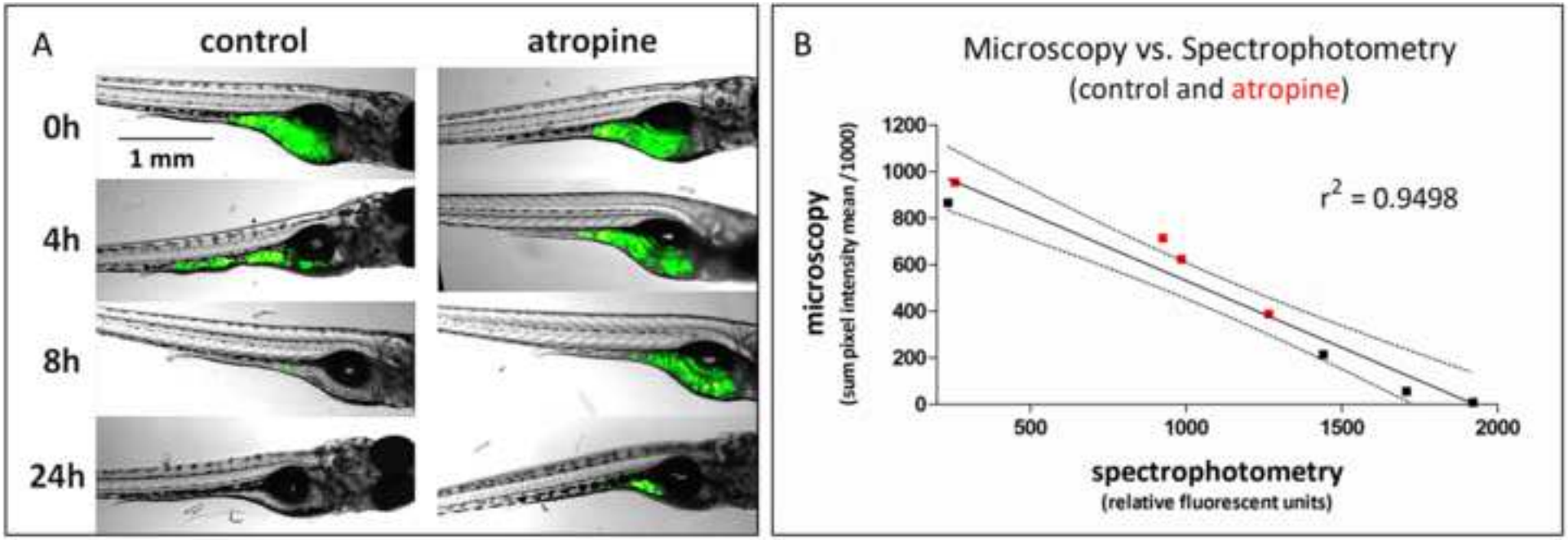

Figure 2

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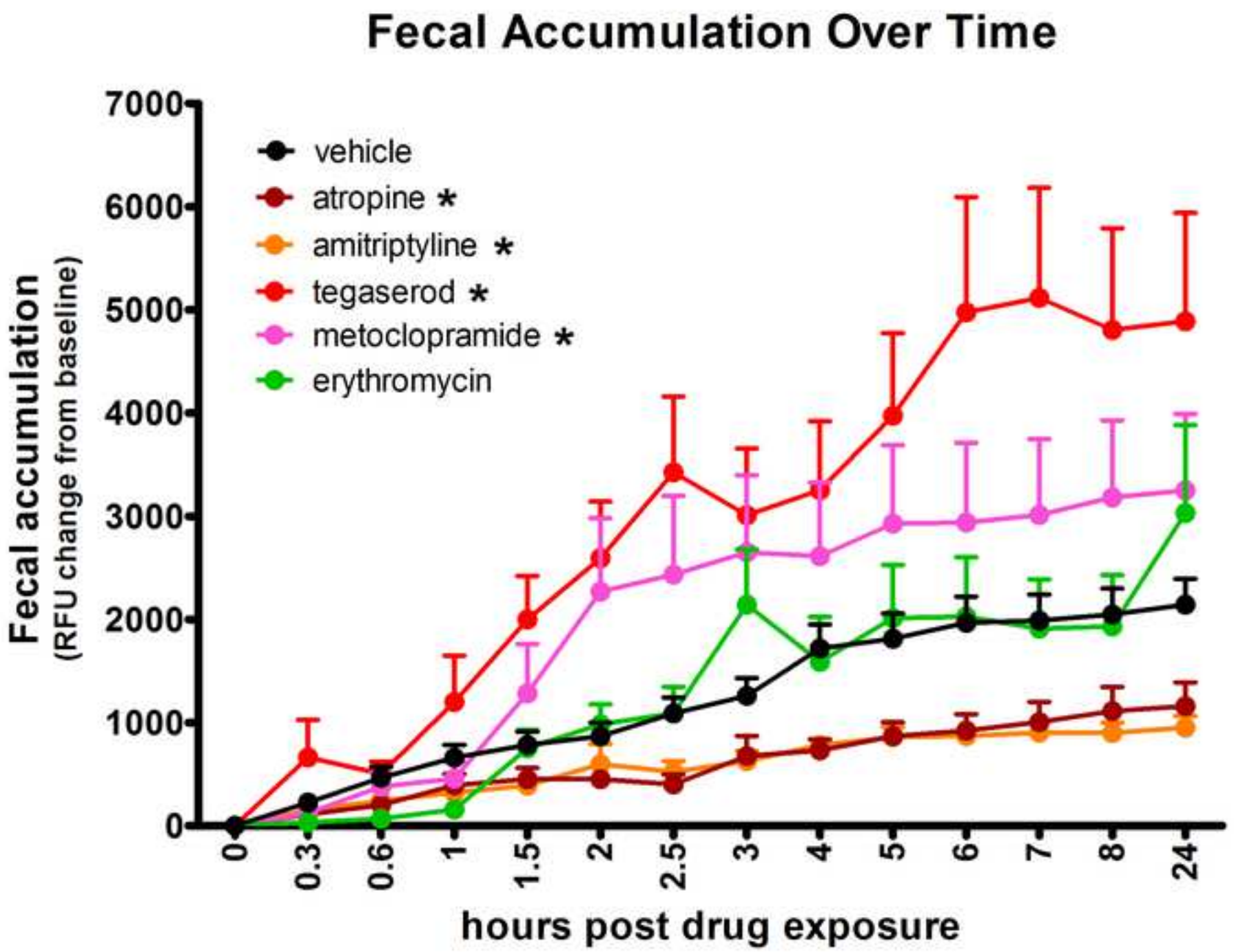

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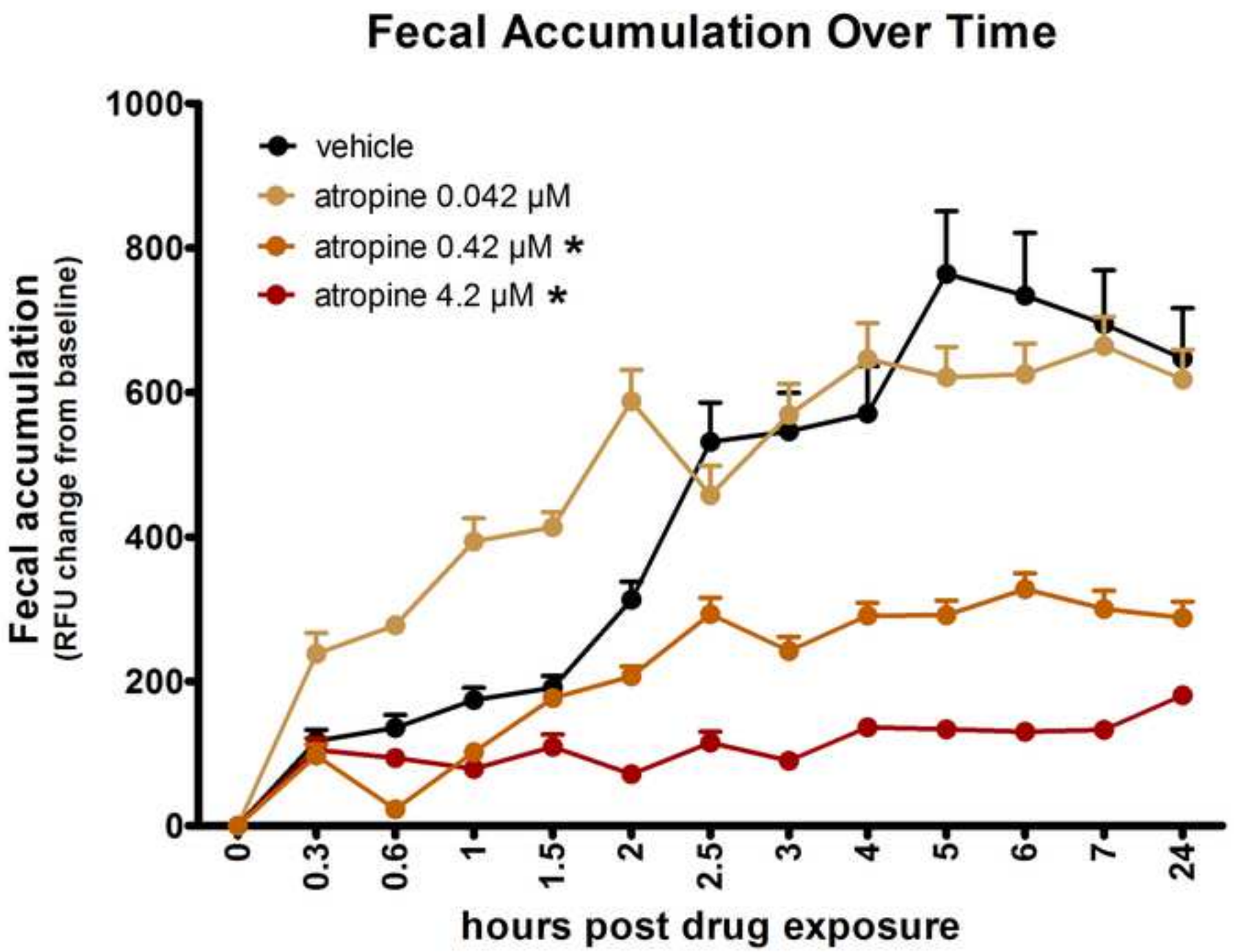

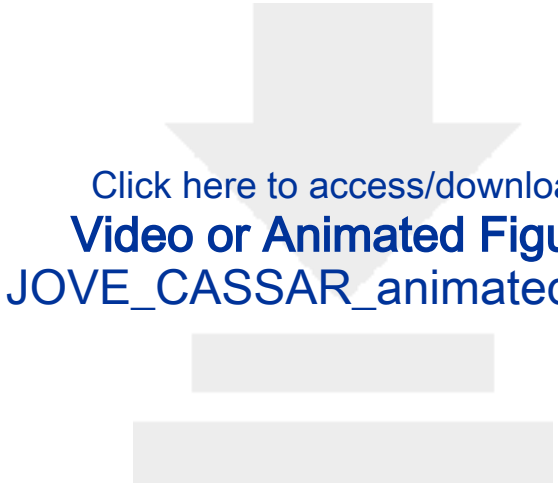

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Table

Table 1: GI activity of 24 compounds in mammals and fish		expected based on active 18	not active 6
zebrafish assay	*active*	*10*	*1*
	not active	*8*	*5*
Positive Predictive Value =		90.9	
Negative Predictive Value =		38.5	
Sensitivity =		55.6	
Specificity =		83.3	

Table of Materials

Click here to download Table of Materials JoVE_Materials_SCassar.xls

Name of Material/ Equipment	Company	Catalog Number
Wild type zebrafish breeding pair	Various sources - for example ZIRC (Zebrafish International Resource Center)	ZL-1
1.7-liter Breeding Tank - Beach Style Design	Tecniplast	1.7L SLOPED
Instant Ocean sea salt	Intant Ocean	SS15-10
First Bites larval fish food	Hikari	20095
Yellow-green (505/515) Fluospheres	Invitrogen	F8827
V-bottom 96-well polystyrene microplates	Thermo Fisher Scientific	249570
Atropine	Sigma Aldrich	A0132
Amitriptyline	Sigma Aldrich	A8404
Tegaserod	Sigma Aldrich	SML1504
Metoclopramide	Sigma Aldrich	M0763
Erythromycin	Sigma Aldrich	E5389
Spectramax M2e microplate reader	Molecular Devices	Spectramax M2e
SoftMax Pro	Molecular Devices	SoftMax Pro

Comments/Description
Adult wild type zebrafish of AB lineage
Breeding tank
Dehydrated sea salt
Powdered larval fish food
Fluorescent label
Multiwell microplate with V-shaped bottom in each well
A multi-well plate spectrophotometer capable of fluorescent excitation and emission detection.
Software for spectrophotometer data acquisition

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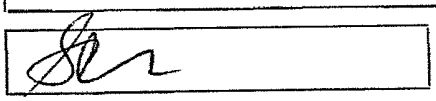
Signature:	 Date: 19-Jul-2018

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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.

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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

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

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.

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High-throughput measurement of gut transit time using larval zebrafish

Transcript for animated figure (JOVE_FINAL_SCASSAR)

- 0:00 to 0:10
  - On the day of the assay, larvae are fed food containing a fluorescent label. And as they eat that food, the label will accumulate in their gut.
- 0:11 to 0:20
  - After feeding, they are washed away from the food, and each larva is transferred into a well of a multi-well plate.
- 0:21 to 0:30
  - The well has a conical bottom to it, so that when fecal matter is voided, it falls to the center of the well.
- 0:31 to 0:40
  - Now remember, there are many larvae in the multi-well plate that can be monitored simultaneously using a plate spectrophotometer,
- 0:41 to 0:50
  - , which measures the amount of voided fecal matter, and as more is voided over time, the signal increases accordingly.
- 0:50 to 1:00
  - In this way, we have a high-throughput method for measuring gut transit.