We thank all authors, as well as the editorial team at JoVE for the time and care with which this manuscript was reviewed. We have made substantial revisions to the manuscript, and believe that we have addressed most, if not all, of the reviewer’s concerns. The major revisions to the manuscript were as follows:

1. Several reviewers noted that the protocol should have more detail, and be easier to follow. We have expanded greatly the protocol section.
2. The editorial board notes that we were not allowed to specify Ethovision, the name of the tracking software. This is unfortunate, because without directing the reader to the name of the software, the steps (many of which are embedded in the Ethovison platform) do not make sense. We have instead given a general approach for tracking, and stated that we use proprietary software, but freely available versions do exist. We hope this change makes it easier for labs that do not have access to Ethovision to perform these experiments.
3. Several reviewers requested more information on the tank, and the setup. Also, we had previously stated that our tanks were purchased from Aquaneering, yet we had to take this out also because Aquaneering is proprietary. To make clear the set-up and the tanks, we have added a new figure with dimensions of the tank, and a schematic of set-up. We hope this helps.
4. A few reviewers requested details on the statistics we used, including tests for normality and power. We have added a statistics section at the end of the protocol, explaining that the experimenter should test their data for normality, and, depending on the result, which sets of statistical measures should be used.
5. We have expanded the introduction and discussion to place this assay in the wider context of the literature, of other available assays for monitoring stress, and with ecological relevance of the approach detailed here.

Whereas the 4 points above represent major changes, detailed responses to each reviewer’s concerns can be found below.

**Editorial comments:**  
Changes to be made by the author(s) regarding the manuscript:  
*1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

We have read through the manuscript and ensured no typographical or grammatical errors.

*2. Please note that numbering of institutional affiliation should follow the order of authors. First author gets 1, next author with different affiliation gets 2, etc., following from first to last. Please label/number the institutional affiliation of each author sequentially.*

The author list has been reordered in accordingly

*3. Please provide an email address for each author.*

Done

*4. 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: Aquaneering, Ethovision, ViewPoint, etc.*

We have removed all cases of the name Aquaneering, Ethovision and ViewPoint.

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

All pronouns have been removed from the protocols section.

*6. 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. 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.*

We have revised the protocol section to only include action steps.

*7. 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.*

We have revised the protocol to add significantly more detail. Moreover, we have now added a statistics section detailing how to run post-behavior analysis.

*8. 1.2: Please specify recommended width of the container.*

We have now added the dimensions of the tank we use in the manuscript, and have also included a diagram of the tank in figure 1A. The drawing in Fig. 1A is annotated with approximate lengths.

*9. 1.3 and 1.4: What is the type of infrared lights? What is the size of a white board? Where is the camera located? Please add such details. A schematic showing the setup may be helpful.*

We have specified that the lights are light emitting diodes (LED), and have noted the wavelength range. We have also included details for the size of the diffuser board, and the position of the camera. We hope these additional, together with the diagram of the setup in figure 1, will address these concerns.

*10. 2.1: How many test subjects are transferred? What tank is used?*

We have now made clear that only one test subject at a time is transferred. We have also specified that approximately 10 adult fish per experimental condition should be used, in accordance with what is typically done in the literature.

*11. 2.3: Does novel tank setup refer to the tank prepared in step 1? Please specify.*

We have added the phrase “…1.8 L trapezoidal plastic assay tank (referred to as the ‘novel tank’)…” in step 1.2 to make clear which tank we are referring to.

*12. 3.2: Please specify the type and concentration of drug to be used in the protocol. Please specify the vehicle solvent used.*

We added the sentence, “If the drug is not dissolvable in water, use dimethyl sulphoxide (DMSO) as a solvent” in section 4.1.

*13. 3.5 and 3.6: How about controls? Are they going through the same procedure? Are controls and drug-treated fish put in the same novel tank?*

We have added the sentence, “Repeat steps 2.4 to 2.7 for each adult until all animals in both experiential and control conditions have been tested” to the end of section 3 to make clear fish are tested individually, but both control and experimental fish undergo the same protocol.

*14. 4.2, 4.5, etc.: Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.*

Done

*15. Please include single-line spaces between all paragraphs, headings, steps, etc.*

Done

*16. 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.*

Done. We optioned to highlight the drug treatment, since it incorporates parts from section on novel tank diving alone (i.e., drug treatment is just two rounds of novel tank with drug treatment in between the two trials.

*17. 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.*

Done

*18. 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.*

Done

*19. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.*

Done

*20. Figure 1B and 1C, Figure 2C: Please change “sec” to “s” for time unit.*

Done

*21. Figure 2C and 2D: Please define error bars in the figure legend.*

Figure 2 is now Figure 3. We have included the sentence,” Error bars represent s.e.m.”.

*22. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.*

We have reformatted the in-line references to follow the requested format.

*23. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. See the example below:  
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).*

We have reformatted the references to follow the format list above.

*24. References: Please do not abbreviate journal titles.*

**Reviewers' comments:**  
  
Reviewer #1:  
Manuscript Summary:  
*Following below is the comment about the manuscript entitled "Behavioral approaches to studying innate stress in zebrafish". The authors present an adequate description of the experimental procedures of a protocol widely used in the literature. This article has significant value in this research field, since zebrafish is becoming a popular and useful animal model for behavioral neuroscience studies. Furthermore, an adequate compilation of data is always helpful and allows to have summarized the results accessible in the current literature. The manuscript is well written and may be recommended for publication in this journal.*

We appreciate this reviewer’s favorable assessment of our work, and we thank them for the thoughtful suggestions for improving the protocol. We have addressed all comments below, and feel that the article is stronger now.

Major Concerns:  
For revision, the following changes are recommended:  
*1) The effects of buspirone in zebrafish is well known. I suggest adding these references to corroborate with the results observed in the manuscript.*

Thank you for this suggestion. As the reviewer notes, we and others have used buspirone. These references have now been added, along with a brief statement acknowledging the long-standing use of buspirone in zebrafish research.

*2) The protocol proposed by the authors is not usual. Normally, the zebrafish are exposed in beaker containing the treatment and subsequently undergo the behavioral test. Why do authors propose this new methodology? Is there an advantage?*

We thank the author for bringing this to our attention. In the past, we have performed drug treatment followed by direct assessment of novel tank behavior, as is typically done in zebrafish research. We have begun, however, to perform a ‘wash-out’ period to ensure that only trace amounts of the drug is transferred into the novel tank with the fish. This step may not be necessary, and we now (i) give rationale for the additional step and (ii) acknowledge that the step may be skipped if the experimenter wishes. These details have ben provided to the reader in the note following step 4.6.

*3) I appreciate the observation in the item 2.2. But I suggest adding the randomization and blinding procedures. Masca et al. (2015) and Gerlai (2018), examining the problems of reproducibility in biomedical research, state that one of the main reasons for irreproducible research is the lack of blinding/masking. The author did not describe that randomization was performed to allocate the animals to the treatment groups (control x buspirone). What was the method used for random allocation in the treatment groups? Were experimenters blind to treatment? Were data analysts blind to treatment? These questions need to be addressed and clearly stated in the methods section.*

Thank you for bringing this to our attention. We overlooked these details. Experiments were run blind to drug condition (i.e., the experimenter did not know whether they were administering placebo or drug solution), and were randomized. These steps are now included in the revised protocol in steps 3.3 and 4.3.

*4) I suggest that the authors review the ARRIVE guidelines (Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 2010 Jun 29;8(6):e1000412) to improve the description of the methodology.*

We thank the reviewer for bringing these guidelines to our attention, as we had previously not been aware. These guidelines have been helpful to us in re-writing the manuscript to address the reviewers concerns.

*5) The statistical section is lacking in the manuscript. Did the author verify the distribution of the data before choosing the statistical test? The authors should improve the description of the results. Statistical data must be provided (F, degrees of freedom, p values). Moreover, the number of animals in each test is lacking.*

Thank you for this point. We have added a section to the protocols on statistical methods to use, and state that tests should be performed for normality. In the case that data are not Gaussian, non-parametric statistics should be used. A Gaussian distribution of the data from both Fig. 2 and Fig. 3 (previously Fig 1 and 2) was confirmed using a Shapiro Test, and thus parametric paired t-tests and analysis of variance (ANOVA) were used. We have also added a statistics section, in which we describe testing for normal distribution. In this section, we specifically state the tests use, and, depending on the results, which statistical measure we suggest. In cases were data are not normally distributed, we suggest a Wilcoxon sign rank and a Kruskal-Wallis non-parametric ANOVA.

Reviewer #2:  
  
Manuscript Summary:  
This manuscript provides a method to measure stress behaviorally in adult zebrafish. In innate state, zebrafish prefer the bottom half of a tank in a stressful state. Novel tank test is a wildly used method to study the stress/anxiety in zebrafish. This study described the use of the novel tank test as a method to study innate stress, and provided a detailed procedure for stress-related research.

We thank this reviewer for their comments and suggestions on how we may improve the manuscript. We have addressed all reviewer concerns.

Major Concerns:  
After reading this paper, I think the following should be addressed:  
  
1. In line 83, "Place a board to cover the sides of the to minimize extraneous moving stimuli", the expression is not clear.

Thank you for this. We meant to say that we wish to visually restrict the fish so that it cannot respond to movements in the room, such as movements by the experimenter or other factors. We have tried to clarify this point. We have also not added a cartoon diagram of the set-up.

2. In line 90, what is the size of 1.8 L plastic tank used here, the length, width, depth? If the plastic tank have short width are preferred to reduce movement, how to certain the tank width you use is adequate.

We have added the dimensions of the tank used in the manuscript and have also added an annotated diagram of the tank on Fig 1.

3. In order to conduct novel tank test, how to choose a suitable tank size?

We have tried a number of tanks, including 1 L trapezoidal tanks, 1.8 L trapezoidal tanks (as the one described in the protocol), and rectangular tanks. In our hands, these options work, and thus any tank roughly 1-2 L in volume will suffice. We have added these details in the manuscript in the note of section 1.2.

4. Is there any influence about the initial location where the zebrafish was put into the tank on the results? Is there any evidence?

In our hands, there is no difference. Regardless of where the adult initially is, they reposition themselves to the bottom of the tank immediately.

5. The manuscript was not well written. The author need to carefully check the article.

We have proof-read the manuscript and fixed typographical and grammatical errors.

Reviewer #3:  
  
Manuscript Summary:  
*The manuscript perfectly describes the novel tank test. Despite this test is widely used in the zebrafish research, at least at my knowledge, no detailed methodological videos are published. Since the editorial politics of JoVE clearly states that "...publish expanded descriptions of techniques that have previously appeared in results-based journals", my reviewer report is for acceptance of this manuscript.*

We thank the reviewer for their time in providing feedback of the manuscript. We have added a section of the ecological relevance of the assay. This is an important advantage of the novel tank test, since the behavior is an innate one. We have added these details, and believe it helps the manuscript.

*Minor Concerns:  
As a suggestion, in the discussion, author can discuss some points related to ecology approach of these innate behavior.*

A short comment on the ecological context has now been added to the discussion. We have compared the ecological relevance of this assay with others that we have used, such as electric shock, and made the point that since this is an innate behavior, it is likely more similar to what behavior would be in the wild.

Reviewer #4:  
  
Manuscript Summary:  
*The manuscript describes a simple method to measure stress behaviorally in adult zebrafish. Assessment of the stress was coupled with pharmalogical drug, buspirone.*  
Major Concerns:  
However, the current stage of the manuscript is not suitable for publication. The authors need to improve the manuscript so that the behavioral protocol for measurement of stress in adult zebrafish is reliable. The following are some of the suggestions for improvement:

1) Introduction was not really relevant to the protocol. The introduction can be improved if the authors describe the importance of measurement of stress behavior for different field. Therefore development for standardize and reliable behavior protocol is important.

We believe that the sentence “the importance of measurement of stress behavior for different field” means that we should explain to an unfamiliar reader why assessing stress in fish models is important. If so, we have addressed this by including the sentence, “Despite these critical findings, much remains unknown about genetic and neuronal regulation of stress. As such, many stress related disorders suffer from a lack of therapeutics.” We believe this provide a reader unfamiliar with stress research sufficient rationale for examining stress in model systems.

We also acknowledge that the description of the HPI axis in zebrafish is not directly relevant to the protocol in this manuscript, but we believe highlighting the conserved physiology between fish and mammals is important in validating the zebrafish as an animal model. We vacillated on whether to remove it, but in the end, we’ve decided to keep it. We hope this is acceptable and that this addressed this reviewer’s concerns.

2) Method was not clearly stated. There are several questions should be addressed by the authors such as how the stress was induced in the zebrafish, what is the minimum and maximum number of zebrafish can be practically use to achieve statistical power. Measurement of the stress in the zebrafish was conducted in plastic tank, however, Cachat and Levin used trapezoid for the measurement of anxiety-like behavior. What is the author's justification of changing the type of the tanks? Since you are using a plastic tank, the behavioral test should be open field test which only allow you assess the locomotors activity. For drugs treatment, you should have both positive and negative control for further validation of stress response toward buspirone treatment.

* We now have added the sentence, “A power analysis should be performed before experimentation, yet in our hands, an n=10 is usually sufficient to detect statistical significance.”
* The earlier manuscript depicted cubed tanks, though in fact the tanks we use are trapezoid in shape. The error in our drawing has been corrected, and we thank this reviewer for pointing this out.
* We are not sure what this reviewer means by, “Since you are using a plastic tank, the behavioral test should be open field test which only allow you assess the locomotors activity.” It seems this reviewer is suggesting that the material used to generate the tank determines whether the test is an open field test or a novel tank. In our set-up, we record locomotor activity in the y-z direction (i.e., we examine depth). By contrast, open field tests typically examine x-y displacement. We therefore respectfully disagree that because our tanks are plastic, this constitutes an open field test.

3) Representative results: You should clearly state the parameters that can be measured using your protocol to assess stress.

We thank the reviewer for this point. We have added the following sentence to the representative results: “Because innate preference was different between the first and last minute, and not distance traveled, we believe the change in behavior represents a stress response, and not merely a change in locomotor activity.”

4) Discussion is not relevant as it does not thoroughly discussing the protocols in terms of troubleshooting, limitations, precautions etc.

We have added these details to the discussion.

Minor Concerns:  
In general, the manuscript needs to be proofread by native speaker.

We have proof read the manuscript to correct for the very few grammatical errors in the initial version.

Reviewer #5:  
  
Manuscript Summary:  
*This methodology paper describes a protocol to behaviorally and pharmacologically assess stress related behaviors in zebrafish using the common Novel Tank Diving Test. It describes the behavioral, pharmacological, and analytical steps involved.*  
  
*Overall, this manuscript reads well and the protocols are clear. While similar papers describing the behavioral and/or pharmacological methodology for this assay in zebrafish exist in the literature (DOI: 10.1038/nprot.2010.140, ISBNs: 978-1-60761-953-6, 978-1-4939-6002-6, to name just a few), I suppose the most useful contribution would be the video representation of the process.*

We thank this reviewer for their positive assessment of our manuscript, and for their thoughtful critique of the work. We have addressed the comments and concerns below, and believe this has improved the manuscript.

Major Concerns:  
- I am surprised at the rather limited acknowledgement (or citations) for the many studies in the field (behavioral and/or behavioral pharmacology) that use this assay in zebrafish. I suggest the authors include a broader coverage of the literature.

We agree that the manuscript could benefit from a brief discussion of the widespread use and important findings that have emerged from this assay. While we believe that a detailed list or discussion is out of the scope of the manuscript, we have added a brief review of relevant findings. These details have been added to the beginning of the discussion.

Minor Concerns:  
- Line 24: What are genetic lesions? Perhaps the authors meant genetic mutants or knockouts?

This was meant to refer to any genetic aberration (mutation, transgenic insertion, etc.) We have replaced the word lesion with, ‘mutation.’

*- In the "Pretreatment with drug", I suggest adding a "note" stating that appropriate behavioral pharmacological procedures be followed (e.g. ensure proper dosage, administration length/frequency, drug crosses blood-brain barrier, and so on). The 10 minute treatment the authors describe may only apply for a specific drug at a specific concentration.*

We have added that in a note in step 4.5.

*- In the "Video analysis" section, I recommend the authors also note that this protocol is just one of several ways to do the analysis. I use a slightly different procedure in Ethovision XT in my research.*

The section on tracking had to be mostly removed, since *JoVE* does not allow mentioning the word Ethovision, since the software is proprietary. In its place, we have highlighted the typical main steps that one would do to proceed with tracking the animals, and have also included some considerations. We also have a note in this section stating explicitly that other labs may have their own variations to this general approach.

*- Line 211-213: Change "stress response" to "behavioral stress response". This paper is looking at behavioral measures and not physiological/glucocorticoid stress response.*

Done*.*

*- I am not sure if this is common in JoVE articles but a "troubleshooting" section with some common problems and potential solutions could be useful for first-time adopters.*

This is an excellent idea. To see if this was an appropriate section, we reviewed several *JovE* papers, but did not find any (at least that we examined) that had a troubleshooting section. Instead, we have added more general notes to the protocol section, with some cases of troubleshooting advice. We hope that the precautions included in notes and the discussion should suffice in helping solve some of the simple complications.

*- Figure 1C & 1D: I suggest the authors use a different color font to represent the individual data points. The gray data points on the patterned background (last minute) are difficult to distinguish.*

We agree that the individual points in Fig 2 do not contrast well either. We have changed the dots in both figures so that they are more easily visualized when overlaid on the bar graph (i.e, we made them red)..

In compliance with data protection regulations, please contact the publication office if you would like to have your personal information removed from the database.