**Response to editorial comments:**

• Editor re-wrote some of your protocol steps in the imperative tense (as if you are telling someone how to do the technique, i.e. “Do this”, “Measure that” etc.). Any text that could not be written in the imperative tense was added as a “Note”.

> Protocol edits noted without additional change.  
  
• Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

> Manuscript has been proofread.  
  
• JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.  
\*Please include “doi” in the citations according to the following format: (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).]

> DOIs are included for all references.  
  
• Please disregard this comment if all of your figures are original. If you are re-using figures from a previous publication, please 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]."

> All figures are original.  
  
• Acknowledgements: Please list all funding sources for this work in this section.

> Funding source noted.  
  
•Minor formatting notes:  
-Please make units of time consistent. Here use SI units (ie “s” for “second(s)”).

> Units of time are now consistent throughout manuscript, using “s” for “second(s)”. As a note, this differs from the formatting guidelines provided in the “Instructions for Authors” document.

-Figure 1, 2, 3 – Please provide scale bars.

> Scale bars included.

**Reviewers' comments:**

**Reviewer #1:**  
  
-Line 64 - consider change „that electrodes be"

> Sentence changed.

-Lines 67-71 - could you provide some citation(s), or is not it discussion? - A distinct advantage to these approaches is that heart rate is assessed in an intact, living bee, rather than in a dissected specimen. The challenges of these methods include accounting for immobilization or anesthetization of the subject, the need to limit outside variables and stimuli that might alter heart rate, and determining an appropriate delivery method when testing pharmacological agents.

> Citation provided.

-Lines 75-77 - In this protocol, the heart is continually bathed with running physiological saline and test compounds can be dissolved in this solution for application to the subject. - reference?

> Citation provided.

-Because the method you describe is cheap and easy, is it useful to teach students, in universities or even high school?

> Agreed, and noted in discussion.

-The fact that the non-invasive methods provide analysis of non-dissected specimen should be better explained regarding the benefits of the non-invasive methods.

>Additional explanation provided.

-Noninvasive, electrocardiographic methods described by Karel Slama might be mentioned: e.g. see doi:10.1016/S0022-1910(03)00065-9 (Mechanical aspects of heartbeat reversal in pupae of Manduca sexta); doi:10.1016/S0022-1910(99)00208-5 (Extracardiac versus cardiac haemocoelic pulsations in pupae of the mealworm (Tenebrio molitor L.)

> Good point, but Slama’s methods were based on and directly cited Wasserthal. A description of this method and reference to Wasserthal (doi:10.1007/BF00691029) has been added.

-Consider mention that the dorsal vessel has been used as the source of hemolymph for proteomics - from red-eye pupa, emerge bee or adult bee; (more than 20 uL of hemolymph is possible obtain after the direct puncture of the adult honeybee hemolymph), see e.g. DOI: DOI: 10.1007/s13592-016-0437-7 ; 10.1007/s13592-013-0230-9 ; DOI: 10.1007/s13592-012-0190-5 ;

> It is unclear how this relates to the protocol being presented in this manuscript, as hemolymph extraction is not a part of the procedure. The described protocol results in a dissected abdominal wall that is buffered with an isotonic solution. Respectfully, no mention of this has been included.

-Line 128 - CO2 (N2O is an alternative)

> It is understood that there are a number of alternative anesthesia methods that are available, however, none of these have been tested by the authors other than cooling and CO2.

-Line 316 - "Overall, the goal of this proposal is to provide a simple …"This should be changed, because you discuss what you did. At least I think that „the goal was to… „

> This line has been changed.  
  
I recommend change structure of the paper: Discussion lacks references. Like to see in the discussion - benefits and limitations of your method compared to the other methods including references - some indications are in introduction (consider change introduction and discussion - suggest move some parts that resemble discussion from introduction to the discussion). In introduction, different methods should be mentioned, but not discussed. The cases of suitable use might be better explained.

> Thank you for the comments and feedback. The approach being followed here is to provide the reader with some background on various methodology for measuring insect cardiac activity, and to provide context for the protocol being described. The suggestion to expand upon the discussion has been heeded, and additional descriptions have been added.

**Reviewer #2:**  
  
*Major Concerns:*  
1) The dissection procedure as described, implies that honeybee preparation suffers from loss of hemolymph that may affect the proper function of the heart and the proper response to compounds. For example see lines 137-138:, Using forceps and/or microdissection scissors, remove legs and wings to facilitate dissection of the abdomen , lines 153-154: Gently remove the stinger and any portion of the gastrointestinal tract that remains attached to the dorsal abdominal wall

> Respectfully, the concern being presented here is unclear. The protocol describes a dissection of the dorsal abdominal wall of the bee in order to visualize the dorsal vessel, absent the remainder of the bee. A loss of hemolymph is expected, and the use of an isotonic solution to maintain osmotic balance is described.

2) The dissection protocol is not clear since it does not refer to the fate of the central nervous system, and especially of the abdominal nerve cord, which is known to modulate heart rate in honeybees [see lines 343-345: Schwab, E. R., Chilson, R. A. & Eddleman, C. D. Heartbeat Rate Modulation Mediated by the Ventral Nerve Cord in the Honey-Bee, Apis-Mellifera. J Comp Physiol B-Biochem Syst Environ Physiol. 161 (6), 602-610, doi: 10.1007/Bf00260751 (1991)].

> A description of the fate of the abdominal nerve cord and its effect on heart rate has been included.

3) The counting protocol based on hand tally counter seems to be not accurate for the high beating frequency of the honeybee. This also implies that the counting protocol may not be practical for and/or insufficient to successfully measure intense cardio acceleration.

> We disagree with this observation. As noted by Schwab (doi: 10.1007/BF00260751) and Papaefthimiou (doi:10.1016/j.jinsphys.2010.11.022), as well as our own observations, severing the ventral nerve cord greatly reduces the frequency and magnitude of dorsal vessel contractions and effectively stabilizes heart rate. The goal of this protocol is to record frequency, and not magnitude, of contractions. While a hand tally counter and visual observations would likely be insufficient to record heart rate in a living, intact adult bee, it has proven more than sufficient to measure heart rate in this protocol.

4) Measuring heart rate, lines 188-189: (for example: record the number of contractions during a 30 s period and multiply the results by 2). This approach attempted by the authors may introduce error, since the interspike interval, or period of heartbeat is assumed to be stable in this case.

> This approach has been removed from the protocol.

5) The changes in average bpm in either DMSO or octopamine (Fig. 4,5) are confusing without giving the mean heart rate value in bpm or mean heart beating frequency in Hz. A percentage increase in basal heart rate could also be useful.

> Results have been modified to reflect a percentage increase in basal heart rate.

6) Lines 271-272 and 278 (one-way ANOVA with Dunnett's multiple comparison test). The statistical analysis used by the authors requires that data follow normal distribution. The authors should state that they checked data for normality with the appropriate test. If data do not pass normality, authors should consider using a non-parametric analysis for non-normally distributed data.

> Data passed a D'Agostino & Pearson normality test, however, it is likely that not all data collected in this manner will follow a normal distribution. A non-parametric analysis would probably be a more widely-applicable method to recommend and this protocol has been updated to reflect that.

7) Discussion section: A comparison of the major outputs of the bioassay such as: i) heart rate in bpm and, ii) response to DMSO and octopamine, with previous studies in honeybee and other insect hearts is missing. Establishing the reliability, accuracy and sensitivity of the bioassay which is crucial for supporting its efficiency and usefulness is also absent.

> Thank you for the comments and feedback. As suggested, the discussion section has been expanded to include a comparison of outputs to other studies cited.