**Editorial comments:**  
Changes to be made by the Author(s) regarding the written manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

**This has been done to the manuscript.**

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

**Both Estep et al. 2016 and Sanscrainte et al. 2018 are** **published under a creative commons license (CC BY 4.0 -** <https://creativecommons.org/licenses/by/4.0/>**) and are therefore allowed to be re-printed. This information has been uploaded in the resubmission and all figures that use previously published data cite the original source appropriately.**

3. Figure 2: Please include a space between numbers and their units of the scale bar (i.e., 100 µm). Please use colored arrows for contrast.

**These changes have been made to the figure.**

4. Figures 4 and 5: Please shorten the figure legends. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

**Figure legends have been shortened and text moved to the appropriate sections.**

5. Please revise the title to be more concise.

**The title has been changed to “Methods for reproducible microinjection of dsRNA and**

**oviposition bioassay in mosquitoes and house flies”.**

6. Please provide an email address for each author.

**Neil D. Sanscrainte -** [**neil.sanscrainte@ars.usda.gov**](mailto:neil.sanscrainte@ars.usda.gov)

**Christy M. Waits-** [**christy.waits@ars.usda.gov**](mailto:christy.waits@ars.usda.gov)

**Christopher J. Geden -** [**chris.geden@ars.usda.gov**](mailto:chris.geden@ars.usda.gov)

**Alden S. Estep -** [**alden.estep@ars.usda.gov**](mailto:alden.estep@ars.usda.gov)

**James J. Becnel -** [**james.becnel@ars.usda.gov**](mailto:james.becnel@ars.usda.gov)

7. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

**These changes have been made to the text.**

8. 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. For example: Kimwipe, Calf Manna, etc.

**These changes have been made to the text.**

9. 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. Please move the discussion about the protocol to the Discussion.

**These changes have been made to the text.**

10. 3.2.4: Please specify the size of the small slit. How many females are transferred into one cup?

**These changes have been made to the text.**

11. 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.

**These changes have been made to the text.**

12. 4.1: Please break up into sub-steps.

**This step has been altered and now conforms to the format.**

13. 4.3.4: There is no step 6.3.3. Should it be 4.3.3?

**This has been corrected.**

14. 4.3.6: There are not steps 6.3.5 and 6.3.2. Should them be 4.3.5 and 4.3.2?

**This has been corrected.**

15. 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.

**This has been done to the manuscript.**

16. 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.

**This has been done to the manuscript.**

17. 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.

**This has been done to the manuscript.**

**Reviewers' comments:**  
  
**Reviewer #1:**  
Manuscript Summary:  
The article describes a method for the injection adults with dsRNA, for the purpose of knocking down expression  
  
Major Concerns:  
Nothing major.  
  
Minor Concerns:  
The nature of the hole for transfer of injected adults between cups would have been useful.

**This change has been made to the text.**

I have tried to attach an annotated PDF with comments and sections highlighted where i think small improvements and clarifications could be made - there was no facility to do so I have cut and pasted the comments from my PDF file below:  
  
Page: 3  
line 67 - could do with a bit of expansion - I doubt these applications will be familiar to most, and certainly not how this technique feeds into those

**These changes have been made to the text.**

line 80 'exposure to CO2' is vague, how long? what dose? In my experience excessive CO2 exposure itself can cause a reduction in egg output, at least when given to smei-gravid/gravid females.

**A change has been made to the text. In this procedure, only non-gravid mosquitoes and flies**

**are knocked down with CO2.**

Page: 4  
line 101 this needs more detail - make or type of puller at a bare minimum. Can it be made more generic for use with other types of puller? What are the desirable physical characteristics of the pulled needle -OK, scap this comment, i've now seen the table at the end. Maybe just refer here in the first instanc eto the presnce of the table describing all equipment

**This change has been made to the text.**

what does this mean? meniscus movement of the liquid in needle?  
 **Yes, meniscus movement of the liquid in the needle. This has been clarified in the text.**

Page: 6  
lines 231 and 232 these should say 4.3.5 and 4.3.2 (not 6.3.5 etc.)

**This has been corrected.**  
  
**Reviewer #2:**   
Manuscript Summary:  
In the article titled "Enhanced methods for dsRNA delivery by microinjection: measuring effects of gene silencing on survival and oviposition of Aedes mosquitoes and house flies," Sanscrainte and colleagues provide a detailed protocol for injecting dsRNA into the hemolymph of Aedes aegypti mosquitoes and Musca domestica flies, as well as methods for oviposition assays to assess effectiveness when using RNAi targeting genes that influence ovarian development. Although other articles published in JoVE have presented microinjection protocols, this topic is worthy of additional detailed methodology in the literature given the difficulty of protocol transfer between labs. Moreover, I believe this is the first JoVE protocol to include microinjection of house flies and the first to describe on oviposition bioassay, which adds novelty.  
  
In general, the manuscript was well-written and free of grammatical errors, and the authors did a nice job explaining important details that are often overlooked. Nevertheless, I recommend that the authors make several changes that would make the paper stronger and more useful to its target audience. I outline my recommendations below by section.  
  
Major Concerns:  
PROTOCOL  
\*Lines 96-127: In steps 2.1-2.4.3 of the protocol, there is no information about dsRNA preparation or quantification. These steps are critical to successful RNAi and should be described in sufficient detail to complete an experiment. Otherwise the authors' claims in the abstract that they are providing a "quantitative microinjection protocol that ensures accurate delivery of specific doses of dsRNA…" are misleading.

**The design and production of dsRNA for microinjection into dipterans has been detailed in**

**[Estep et al.]5 and [Sanscrainte et al.]9. References to this as well as a description of how to**

**quantify dsRNA have been added as 2.1.**   
  
Minor Concerns:  
ABSTRACT  
\*Line 47: the word "most" should be removed. The authors identify effective locations for delivery, but did not compare injection sites, so there is no data supporting that the injection sites are the "most effective" ones.

**This change has been made to the text.**

INTRODUCTION  
\*Lines 53-55: In the sentence starting with "Oral uptake of dsRNAs…" information on the genes targeted in the cited studies would be appreciated. In line 55 please edit the last part of the sentence to say "…after feeding dsRNA to adult Ae. aegypti in sugar meals." When I first read the sentence, I interpreted it to mean that the phenotypic effects of RNAi in larvae vanished in the adult after sugar feeding.

**These changes have been made to the text.**

\*Line 69: Please briefly define "biorationals." Some readers will be unfamiliar with the term.  
 **These changes have been made to the text.**

PROTOCOL  
\*Lines 96-127: When describing needle/solution preparation the authors do not mention backfilling their needle. If their system does not require that step, perhaps a sentence or two addressing backfilling is warranted since some commonly used microinjectors have unusable dead space between the plunger and the injection solution that must be filled with oil (which won't mix with the injection solution).

**This change has been made to the text.**

\*Lines 113-114: Recommendations or examples of "desired dose" with citations would be worthwhile, particularly for labs wanting to attempt the protocol for the first time.

**This change has been made to the text.**

\*Line 133: The word "mosquitos" should be "mosquitoes."

**This change has been made to the text.**

\*Line 135: The placement of "(Fig 2A)" should be earlier in the sentence after "mesokatepisternum." In its current location, I expected to see an image of the needle tip puncturing the cuticle. Similarly, "(Fig 2B)" should be moved to follow "mesopleuron."

**These changes have been made to the text.**

\*Line 139-140: I just wanted to make sure that I understood this correctly. Are the authors saying to insert the tip of the needle through the midline of the insect? That seems deep. In my experience, shallower injections in mosquitoes lead to better survival. However, I noticed below that the authors typically get > 97% survival, which is impressive. Do the authors see variation in survival depending on species of mosquito injected and or depth of injection? Perhaps a sentence or two about injection depth are warranted, particularly if the authors find that deeper injections improve knockdown of expression.

**The reviewer is correct as to the depth of injection; shallow injections frequently result in the**

**injected solution beading back out of the insect. A note has been added to the method to ensure that the importance of depth of injection is understood.**

\*Lines 143-144: Do the authors find that for mosquitoes multiple injections of lower concentration dsRNA get better results than single injections of more highly concentrated dsRNA? If so that would be useful information.

**The use of multiple pulses (during a single injection event) is simply to deliver a desired dose based on our starting concentration. We have no data as to the effect of further concentrating the dsRNA and reducing the net injection volume, but no reason to suspect it would not be as effective.**

\*Line 153: After the word "tulle" please add "or netting." Some readers will have no clue what tulle is.

**This change has been made to the text.**

\*Lines 153-155: For the sentence beginning with "After mosquitoes and flies…", is the cup inverted for a reason rather than the cotton ball being placed on top of the tulle? An image would be helpful to understand what the authors mean.  
 **The inversion of the cup on top of the sucrose cotton attempts to aid in survival by providing**

**easier access to a sugar meal (see Isoe et al. 2011). A note has been added to the method to make this clear.**

\*Line 206: Is this supposed to say black cloth instead of "black cotton?"

**This has been corrected.**

\*Line 209: An image at the end of the sentence would be appreciated.

**A new image has been added and called out as suggested.**

\*Line 225: The sentence should read "Repeat step 4.3.3…" Also in that line, the authors should specify quantitatively what they mean by "severe outlier."

**This change has been made to the text.**

\*Line 231: The sentence should read "…value obtained in step 4.3."

**This change has been made to the text.**

DISCUSSION  
\*Lines 312-313: The sentence beginning with "Delivered volume…" should be edited to say "Delivered volume and concentration are critical parameters that allow …"

**This change has been made to the text.**