Dear Editor and Reviewers,

We would thank you for your kind reviews and constructive comments on our manuscript. We have carefully revised the manuscript to address your comments and suggestions. The following is a point-by-point account of our responses to your comments.

Editor's comment:

Changes to be made by the Author(s):

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.

***Response:*** *We have done all necessary proofreading, including spelling and grammar issues.*

2. Unfortunately, there are a few sections of the manuscript that show overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 47-49, 79-80,

***Response:*** *We have rephrased those two sentences.*

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

***Response:*** *We have checked and confirmed that there is no use of “could be”, “should be” or “would be” through the protocol.*

4. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

***Response:*** *Necessary changes have been made at line 227 and 232.*

5. Please add more details to your protocol steps. 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.

***Response:*** *We have added more detailed description about PCR, LIC cloning, transformation, protein purification and crystallization, and structure determination. We also added the reference for LIC cloning strategy.*

6. 1.1: How is the PCR done? What is the recipe? What are the primers used?

***Response:*** *Details of primer information and PCR procedure has been added in section 1.1.1 and 1.1.2, respectively.*

7. 1.2: How is the transformation done?

***Response:*** *Transformation procedure has been elaborated in section 1.2.*

8. 1.3: What happens after centrifugation? What volume is used to resuspend? What are the sonication settings?

***Response:*** *Additional details have been added in a new section 1.4.*

9. 1.4: How is this done?

***Response:*** *Details about HisTrap purification has been added in section 1.5.*

10. 1.5: How is the reaction done? How is the purification done?

***Response:*** *TEV protease cleavage reaction and post cleavage purification steps have been elaborated in section 1.6 and 1.7.*

11. 2: Where are the trays incubated? What conditions?

***Response:*** *Incubation conditions have been added in section 2.3.*

12. 3: What is done here? Do you want this to be filmed?

***Response:*** *We have done crystal fishing and freezing, in-house x-ray diffraction for crystal pre-screening, data collection at synchrotron, structure determination and model building on computer here. We want to film most parts except the synchrotron data collection.*

13. There are not enough details in any of the protocol steps to be filmed.

***Response:*** *The following steps/techniques could be filmed: PCR, transformation, cell culture, centrifugation, sonication, protein purification using Akta system, SDS-PAGE, Nanodrop for concentration measurement, crystal screening robot, crystallization incubator, crystal dye checking, crystal fishing and freezing, in house x-ray diffraction and data collection, structure determination on computer, and modeling using 3D glasses.*

14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

***Response:*** *We have re-written the discussion in three paragraphs. We have added information about critical steps, troubleshooting, limitations and future applications of the technique.*

15. Much of the current discussion should be moved to the representative results.

***Response:*** *We have edited the discussion section and moved necessary information to the representative results.*

16. Please do not abbreviate journal titles.

***Response:*** *The journal titles in the references section is abbreviated based on the endnote style file provided by JoVE. Also several recently published papers from JoVE follow the same style. Please provide us updated style file, in case full journal names are mandatory in the references section.*

17. Figure 1: Please include a space between the numbers and the units. Please use kDa instead of KD.

***Response:*** *We have changed accordingly.*

Reviewers' comments:

**Reviewer #1:**

Manuscript Summary:

The manuscript by Nayak et al. reports a generalized protocol for protein expression, purification and structural determination by x-ray crystallography. They use this protocol to solve the crystal structure of the N-terminal domain from ryanodine receptor (RyR) of diamondback moth (DBM). DBM is a devastating pest destroying cruciferous crops, and RyR has been proven to be a valid target of insecticide. RyR-targeting insecticides are among the most popular pesticides on the market. The elucidation of this structure provides insight into the molecular mechanism of insect RyR and its interaction with insecticides. The generalized protocol will help other readers to repeat the experiments related to protein structural studies using automatic protein purification and crystal screening systems. The manuscript is clear and well written. I recommend that the manuscript could be accepted for the publication after some minor revision.

Minor Concerns:

1. The purpose of this study is to develop methods to elucidate of RyR protein structure, while little information was provided to stress the significance of this work and recently progress. Please add in the introduction;

***Response:*** *Thanks for the suggestion. We have added a new sentence “This is the first high-resolution structure reported for insect RyR, which reveals the mechanism for channel gating and provides an important template for the development of species-specific insecticides using structure-based drug design.” into the introduction.*

2. Please add the primer information for PCR amplification or reference;

***Response:*** *We have added the primer information to section 1.1.*

3. The author did not show how to predicted the crystal structure of PxRyR in Figure 3 clearly, such as with which software and what parameters are set etc.

***Response:*** *Figure 3 shows the crystal structure of the DBM RyR NTD solved by x-ray crystallography using the method described in the manuscript. It is not a structure predicted by modelling software.*

**Reviewer #2:**

Manuscript Summary:

The manuscript describes in detail a protocol for characterising the structure of the N-terminal domain of the Ryanodine Receptor in Plutella xylostella. The authors outline, in clear, easy to follow terms, the methodology used. The representative results and data also come across as easy to follow.

Major Concerns:

No major concerns.

Minor Concerns:

Methods: There is only one change I would like to see, and that would be specifying which transformation technique was used for cloning. Usually I would not consider this something to worry about, but as this is specifically a methods paper, I think it is worth including.

***Response:*** *Thanks for the suggestion. We have added the details about the transformation protocol into the section 1.2 of the protocol.*

Results: I know the Rabbit Ryr PBD ID was given as part of the methods. Would it be worth adding the P xylostella RyR ID as part of the results, or potentially in the caption of figure 3?

***Response:*** *The PDB code for DBM RyR has been added to the results and the legend of figure 3.*

References: Not so much concern, but something that might be worth considering. Qi and Casida (Pesticide Biochemistry and Physiology, 2013) propose that Flubendiamide binding sites may differ across species. One possible application of this method might involve looking at various species to determine what structural differences might affect Flubendiamide binding.

***Response:*** *Thanks for the suggestion. We have added a paragraph in the discussion “Qi et al. found that different families of diamides might bind to distinct sites that are different across species. Using our strategy, one can determine the RyR structures from multiple species and identify the unique elements responsible for the species-specificity.”*

**Reviewer #3:**

Manuscript Summary:

The authors describe the quite standard procedure of expression, purification, crystallization and crystallographic data collection and processing of RyRs fragments and other short protein fragments for that matter.

Major Concerns:

The authors completely ignore a crucial step for the success of the process. Since early 2015 there is ample information on the full length structures of RyR1 and RyR2 from cryo-EM studies, non of which are mentioned in the text. These structures can be very useful, if not crucial, for the design of such fragments considering known domain boundaries and the effects of known domain-domain interactions on the expected stability of such fragments when expressed "out of context".

***Response:*** *Thanks for the suggestion. We have added a sentence in the introduction “The construct was designed according to the published rabbit RyR1 NTD crystal structures and the cryo-EM structural models.” and also all the references for the recent papers of cryo-EM studies.*

Minor Concerns:

It is possible but not always the case that a crystal grown in the initial conditions will provide useful diffraction data. It is worth elaborating on optimization procedures for the crystallization and cryo-protection.

***Response:*** *We have added crystal optimization procedures as a new sub-point 2.6.*

Also, worth mentioning is experimental phasing in cases where molecular replacement is not available.

***Response:*** *We have added a sentence “If the molecular replacement fails, one can consider experimental phasing methods, such as single-wavelength anomalous diffraction (SAD), multi-wavelength anomalous dispersion (MAD), and multiple isomorphous replacement (MIR).” in section 3.4.*

Sincerely yours,

Minsheng You and Zhiguang Yuchi