Rebuttal letter

We are pleased for the opportunity to submit a revised manuscript and appreciate the reviewer's and editorial staff's comments and insights. We were able to address all the concerns and hope that the reviewers and editors find the manuscript suitable for publication in the Journal of Visualized Experiments.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have gone over the text in detail and have removed spelling and grammatical issues.

2. Please obtain explicit copyright permission to reuse any figures (including TOC) 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]."

All the figures were newly created for this paper; data from previous papers were re-used, but the analysis was done specifically for this paper. We do not believe that we need to ask for permission to re-use the data as long as we cite the original paper. In addition, the previous papers were published in Nature Communication, a journal under a generous Creative Commons license stating:
"Under this license users are free to share (copy, distribute and transmit) and remix (adapt) the contribution including for commercial purposes, providing they attribute the contribution in the manner specified by the author or licensor"

and

"Under Creative Commons, authors retain copyright in their articles."

For more details:
https://www.nature.com/ncomms/about/open-access

As such, we have fulfilled the license requirements by citing the paper.

3. Please do not use abbreviation in the title.

TX-MS is not an abbreviation but the name of a specific method. This name is an acronym, but the issue here is that we do specifically refer to the TX-MS method, not to "targeted cross-linking mass spectrometry" in general. We have, however, updated the title in accordance with a reviewer comment and we hope that the new title is permitted.

4. 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.

We are adhering to the requested citation format.

5. Step 4.2: Please specify the code.

The idea of the paper is that this input comes from the user, to provide the opportunity for the user to explore the data in greater detail compared to the original report.

6. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account.

We have removed the embedded figures and uploaded the figures using the editorial manager account.

7. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

We have accompanied each figure with a title and description.

8. 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

We have extended the discussion to cover the request explicitly.

9. Please revise the table of materials to include all essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

We have revised the materials.

Reviewers' comments:
Reviewer #1:
The manuscript as presented is minimal and it was not possible to fully review the work. What could be reviewed was not substantial enough to provide significant help to a non-expert and was poor for an expert. In general, the protocol only provides minimal detail and should be substantially expanded. Few of the steps are clear and the data linked from the manuscript is not easily found.

We have extended the manuscript to provide more help for users. We have also uploaded the needed input files and containers to zenodo.org and provide the DOI (10.5281/zenodo.3361621) in the manuscript.

Specific points are found below, these are not by any means exhaustive.
1) It feels highly disingenuous that they appear not to have cited any previous XLMS software?
E.g. xQuest (Rinner et al., Nat Methods 5(4):315-8, 2008; Walzthoeni et al., Nat Methods 9(9):901-3, 2012), pLink (Yang et al., Nat Methods 9(9):904-6, 2012), ProteinProspector (Chu et al., Mol Cell Proteomics 9:25-31, 2010; Trnka et al., 13(2):420-34, 2014), Hekate (Holding et al. J Proteome Res 12(12) 5923-593, 2013) and Kojak (Hoopmann et al., J Proteome Res 14(5):2190-198, 2015) etc.

We are now citing the papers pointed out by the reviewer.

2) On line 69 they mention that this method is "significantly more sensitive compared to previous methods" highlighting the lack of references to previous methods and without substantiating this claim.

We are referring to the TX-MS paper where we did indeed substantiate this claim to the satisfaction of the reviewers of that paper. The original submission of that paper contained a supplementary note that detailed our performance compared to established tools; however, that supplementary note was left out from the final paper as, through discussions with the Nature Communication editorial staff, we felt that such a comparison could not be carried out objectively as we know our tools better than the established tools. The supplementary note, in short, demonstrated that TX-MS was the only tool able to identify inter-crosslinks between proteins in the most complex samples, human plasma adsorbed onto live bacteria.

3) I cannot properly review step 1 - Run TX-MS as this requires me to sign up with name and organisation. As this is under peer review and to sign up would bypass the anonymous/blind peer nature of this review.

This is rather unfortunate, we sent login credentials for five reviewer accounts to JoVE to be sent to the reviewers, but something must have gone wrong. The login accounts are:

reviewer1 VaayMM1D
reviewer2 XM2CCipW
reviewer3 5rpu4TBi
reviewer4 8UHrPWzq
reviewer5 WG29f6yv

4) On line 94 it states "NOTE: There is example data available on txms.org", this should be included as a supplementary file or directly linked with a doi.

We uploaded data and containers to zenodo.org under DOI 10.5281/zenodo.3361621. The data from the TX-MS paper is also on zenodo.org and can be used as input, under DOI 10.5281/zenodo.3361621.

5) On line 96 they mention uploading two structures - this implies the method is limited to this number. The authors should clarify.

The current implementation is limited to two; we have clarified this in the text.

6) On line 101 it states "NOTE: There is example data available on txms.org" this should be included as a supplementary file or directly linked with a doi.

We uploaded data and containers to zenodo.org under DOI 10.5281/zenodo.3361621. The data from the TX-MS paper is also on zenodo.org and can be used as input, under DOI 10.5281/zenodo.3361621.

7) On line 115 it is not clear if "docker pull 116 malmstroem/jove:latest" a Windows, Mac or Linux terminal command. How is the average scientist meant to realise this? I had to run it in the "Docker Quickstart Terminal" not on the mac terminal.

We have made the instructions clearer by providing instructions for each operating system.

8) On line "Start the docker container: docker run -p 8178:8080 malmstroem/jove:latest" I have to correct 8080 to 8000 to make this command work.

We apologize for this error and have corrected it.

9) "Click on the log-in button on the openBIS Jupyter extension" Jupyter asked for a username/password to "Sign-in" that I have not been provided. (I found " guest/guestpasswd" and "user/user" on docker hub, but why I am searching for these?).

The fundamental issue is that we do require to log in due to the licensing for Rosetta. Rosetta is only free to use by academics, and hence, we need to verify that users are indeed academic. We have made changes to the manuscript clarifying this.

10) On line "Search for the report and click download" - is completely unclear how to do

this. Again I worked it out from the dockerhub page, but that wasn't particularly helpful either. At this point, I stopped as too much required information outside of the manuscript.

We have improved the manuscript to include better instructions.

11) On line 136 is states "We provide a few examples under the Representative results section" - these are not clearly signposted.

We have clarified this statement.

12) Table of Materials - "Two Protein DataBank files of the proteins of interest." - These should be either a supplementary file, linked to a copy with a DOI, or a direct link to PDB. As it currently stands it is an exercise for the reader to work out where the files are.

We uploaded data and containers to zenodo.org under DOI 10.5281/zenodo.3361621. One of these structures is a model and hence is not present in the PDB.

13) Table of Materials - "An mzML data file acquired on a sample where the proteins of interest were crosslinked/Example files available on txms.org." - should be placed with a link to the DOI file or attached directly to the manuscript, I could not find them on the website.

We uploaded data and containers to zenodo.org under DOI 10.5281/zenodo.3361621.

Reviewer #2:

Manuscript Summary:

Studying Protein- Protein Interactions (PPIs) has become very important for understanding biological phenomenon, esp. the origin of diseases. TX-MS, discussed in this manuscript/ video, is a new method for studying PPIs using Jupyter notebook. It combines Chemical Crossslinking (CXL)-Mass spectrometry (MS) with docking methods - bioinformatics tools (usually developed according to each laboratory's special requirements and writing the required Algorithms). Is this method dependant on Linux systems alone? Would that constitute a limitation? Adding docking data and analyzing these is an added step. We have also done such attempts. Will these necessarily add more quality to the results? Refining cryo EM results using CXL data is still time consuming even if one is using sophisticated software like HADDOCK (High Ambiguity Driven biomolecular DOCKing). TX-MS approach uses "Various MS acquisition data such as hrMS1, DDA, and DIA using the notebook using the NGL view".

We appreciate the reviewer's comments. This method is not dependent on Linux per se, but installation on other operating systems is challenging. Docker is solving this problem, providing a way to run the service anywhere. In this particular case, we carry out all the calculations as a service, allowing anyone with a browser to run TX-MS.

The example given describes only use of a Homobifunctionl Crosslinker, that too, only a di NHS crosslinker, which target mainly Lysine r residues and are less useful for studying intermolecular interactions. While it is true that Lysine residues are in abundance in proteins, yet PPI's are not restricted to studying Lysines alone. In the manuscript there is no mention of Heterobifunctional crosslinkers. Reference to A Sinz's work is completely missing-Pl do cross check this statement of mine. No reference is made to StavroX or MeroX (the latter being more suited for MS/MS cleavable crosslinkers), software developed in her laboratory, which help in identifying Intermolecular cross-linking better. For this StavroX/ MeroX software come in handy. Identifying the two crosslinked peptides/ proteins fragments is very difficult and usually only one of these is actually identified. In our recent paper in 2019, we have successfully identified the crosslinked fragments involving two proteins. Does TX-MS have the capability of doing so. For Chemical Crosslinking-Mass Spectrometry- Bioinformatics to emerge as a rapid, routine and reliable technique in hospital situations with large number of patient samples still remains a distant dream indeed.

The current implementation is only for a small subset of cross-linkers. The reason for this is that we have trained the machine learning step on this type of data; it is possible that the model also works for other cross-linkers, but we would like to verify this before making any claims. All software components other than the machine learning model are capable of handling other cross-links, as long as they are not cleavable.

Major Concerns:
Described is only the use of a homo bifunctional crosslinker

We have clarified this in the manuscript.

Minor Concerns:
Is it restricted to Linux systems?

It is not restricted to Linux, but as non-Linux installations are challenging, we solve this issue using docker.

Reviewer #3:
Manuscript Summary:
This manuscript introduces the protocol for TX-MS (targeted cross-linking mass spectrometry), a method capable of determining large complex structures, using a combination of high-resolution mass spectrometry, chemical cross-linking, and high-accuracy structure modeling. This method provides an alternative path to solving structures of protein-protein interactions, without requiring time-consuming structural biology experiments such as X-ray crystallography, NMR, and cryo-EM. The protocol introduced here demonstrates the procedures of running TX-MS and interpreting the results, using the

Jupyter Notebook platform.
This manuscript presents its potential importance; however, it lacks sufficient explanations and details for the users to repeat the procedures. In general, the current manuscript might not be qualified for publication in JoVE. After going through the manuscript, I have the following questions that preclude this work from publication in the current form.

We thank the reviewer for the comments and present an improved manuscript which we hope fulfills the requirements for publication in JoVE.

Major Concerns:
1. The title chosen by the authors is neither clear nor informative. The authors should use more general terms for the audiences to comprehend the key purpose of this work from the title. In the same vein, there are plenty of abbreviations running through the article without definition. It would be much more formal and apt to provide the full name of a term before using its abbreviation in the first instance; for example, TX-MS, XL, etc.

We have corrected this. TX-MS is the name of a method in this case.

2. Several sentences used in the abstract and introduction are vague. For instance, in the sentence "removing one of the major bottlenecks," the authors do not clearly explain what these bottlenecks are. In another sentence, "A graphical report summarizing the most important results," what do the authors mean by "the most important results?" More specific explanations would be appreciated.

We have clarified these vague statements.

3. In the section of PROTOCOL, the authors might over-simplify the steps for the first-time user to run TX-MS. First, there is no 'upload data' option on the page so I tried to click 'sign up' option followed by filling up the information to start with the tool. Unfortunately, the server directed to a strange page showing some texts (might be php codes), so I couldn't continue to review this work, even though I tried different web browsers (Chrome and Internet Explore).

We apologize for the inconvenience and have fixed the registration page. We also provided JoVE with login credentials to be used by the reviewers, but something must have gone wrong, and these were not communicated. The login credentials are:

reviewer1 VaayMM1D
reviewer2 XM2CCipW
reviewer3 5rpu4TBi
reviewer4 8UHrPWzq
reviewer5 WG29f6yv

4. If the authors can provide clear information about the version of the tools, or even the operating system used in this demonstration, that would be very useful. After all, there are many cases where the users cannot repeat the experiment due to conflicting versions of the tools or operating system. Also, is there any requirement for the Jupyter Notebook version to be installed before installing the JupyterHub? The authors should clarify all this.

We have clarified the versions of the tools and operating systems.

5. That would be helpful if the authors can note the way how users can run the Jupyter Notebooks on a public IP server, rather then a private computer using http://127.0.0.1 as the default address.

We have clarified this statement.

Minor Concerns:
Several grammatical errors need to be corrected.

We have corrected spelling and grammatical issues.