Dear Bing Wu and Reviewers,

We wish to thank you for the time spent in reviewing, commenting, and editing our manuscript. We are pleased with the constructive and positive comments we have received and feel that with your feedback the manuscript (and especially the protocol section) will be improved.

The editorial and reviewer comments were few in number, and are addressed below point by point. We look forward to receiving your final decision on the manuscript.

With best regards,

Joerg Standfuss on behalf of all authors

**Point by point Reply to the editorial and peer review comments:**

*Comments in blue; our response in black.*

**Editorial Comments:**

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

A few edits were made to correct minor punctuation errors. As well edits to the equations for clarity.

1. Keywords: Please provide at least 6 keywords or phrases.

Added “Membrane proteins”, “Three-way coupler” and “X-ray free electron laser” to bring the total key-words/phrases to seven.

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

A thorough search shows that the protocol section as written complies with this requirement.

1. 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”

A review of the protocol text shows that the text complies with this requirement.

1. 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.

Changes have been made to the notes (see below) to comply with this requirement.

1. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

We see that our excessive use of notes in the protocol is unneeded, and is distracting for the reader. The following actions were taken to reduce our use of notes:

The note under step 1 was deleted. Information pertinent to the paper was moved into the Starting Material section in the representative results.

The note under step 1.2 was deleted. Information in this note was inserted into the discussion section (second paragraph).

The note under step 1.9 was deleted. Information in this note was moved to the High-speed camera section of the representative results.

The note under step 1.12 was deleted. The information here is not particularly relevant to the method.

The note under step 2 was deleted. Information was moved to the introduction (paragraph 6) and the High-speed camera section of the representative results.

The note under step 2.1.1 was shortened.

The note under step 2.1.2 was deleted.

The note under step 2.2 was edited for brevity.

The note under step 2.2.2 was made into a step (the new step 2.2.3).

The note under step 2.3 was deleted. Information was moved to the Highspeed camera section of the representative results.

The note under step 2.7 was deleted and incorporated into step 2.1.3, and step 2.7.

The note under step 2.9 was deleted and incorporated into step 2.9.

The note under step 2.11.7 was deleted.

1. 2.11.4: Please specify the software tools used to measure the distance.

The phrase “software tools” was replaced with “straight line measurement tool”.

1. Please number the figures in the sequence in which you refer to them in the manuscript text.

The figures have been renumbered according to the order that they appear in the manuscript.

1. Please reference all figures in the manuscript. For instance, Figure 6 and Figure 7 are not described in the manuscript.

References to Figure 7 (now Figure 3) and Figure 6 (now Figure 5) were added in the representative results section.

1. Figure 3: Please explain what the blue arrows represent.

Added “as illustrated by the blue arrows” to the figure legend.

1. Figure 5: Please add panel labels to the two scatter plots. Please change “sec” to “s” for the time unit.

The changes have been made as suggested.

1. Figure 7: Please insert a space between number and its corresponding unit (i.e., 3 µL).

The change has been made as suggested.

1. Table of Materials: Please replace “ul” with “µL”.

The changes have been made as suggested.

1. Discussion: Please also discuss critical steps within the protocol and any limitations of the technique.

Two paragraphs have been added to the discussion section to address these items.

**Reviewers' comments:**

Reviewer #1:

Manuscript Summary:

James et al. introduced an improved protocol for the high viscosity sample inject for time-resolved serial femtosecond crystallography, which is very suitable for the publication at JoVE.

We thank the reviewer for their recommendation.

Major Concerns:

None.

Minor Concerns:

1. Page 5 line 149, suggest change "Bacteriorhodopsin" to "Protein". Authors used Bacteriorhodopsin for the demonstration purpose in the manuscript, but it will be a little confusing as readers may think it just works with Bacteriorhodopsin.

This is a good suggestion as it does suggest that the method is adaptable. The line now reads “Protein crystal sample preparation protocol.”

2. The lipidic cubic phase has been referred to LCP, mesophase and cubic phase in the manuscript, should be consistent.

This is a good suggestion for improving readability. However, there are subtle differences in meaning between the three terms. For that reason, which term we use and where we use them was carefully reviewed. We have changed a few instances of “cubic phase” to “LCP” and deleted one redundant use of the term “mesophase”.

3. Three-way coupler is a very interesting device, author can share more information about its availability, since it can't be purchased through commercial vendors.

The three-way coupler is a custom-made part manufactured at the Paul Scherrer Institute. Currently there is no plan in place to distribute this device, but we certainly do not object to making these available after the fabrication becomes standardized.

Reviewer #2:

Manuscript Summary:

This presented manuscript is very well written, comprehensible, technically sound and certainly of high interest for the community working in the field, as well as for the general readership.

The paper certainly deserves publication.

All experiments described were performed conclusive.

The authors describe a way to slightly optimize LDC sample preparation (3-way mixing) and delivery/stability (camera setup). It is well written understandable and literature is adequately cited and I again recommend it for publication.

We thank the reviewer for their kind remarks and recommendation.

However, it need to be ensured that the protein and other material is specified in all possible detail, as different batches of membrane proteins may significantly vary in quality.

The table of materials contains all material that was used to perform the work outlined in the protocols. Protein and crystallization materials were not included, but may be found in the papers referenced in the Starting Materials section in the representative results.

Although the authors should provide some data or information in the discussion, if and how the protocol can or cannot be adjusted (which appropriate parameters or paranter range) to transfer the protocol to other proteins, different to bacteriorhodopsin and eventual to water soluble proteins and protein complexes, RNA ect.

Modifications are addressed in the discussion section, but are kept general because the variability between samples or viscous carriers is too large. The criteria by which we judge successful sample optimization are listed in the discussion section.

Major Concerns:

none

Minor Concerns:

see above