

Editorial and production comments:

We thank the editors and the reviewers for their time and comments. We have addressed each of the concerns to the best of our ability and detail the changes made below in italics.

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.

Done.

2. Please provide an email address for each author in the manuscript text.

Done.

3. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

Done.

4. JoVE cannot publish manuscripts containing commercial language. 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: Whatman, Talos Arctica equipped with a Gatan K2, etc.

We have removed all examples of commercial language within the manuscript.

5. 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.

Done.

6. The Protocol should contain only action items that direct the reader to do something.

Done.

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

We have removed all examples of personal pronouns within the protocol portion of the manuscript.

8. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

We have strived to ensure that the manuscript is sufficiently detailed.

9. Only one note can follow one step. 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.

We have removed any examples of duplicate notes and/or modified accordingly.

10. Please describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. Data from both successful and sub-optimal experiments can be included.

We have added a sub-optimal atlas in figure 4

11. 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].”

We have not re-used any previously published figures within this manuscript.

12. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

Done.

b) Any modifications and troubleshooting of the technique

Done.

c) Any limitations of the technique

Done.

d) The significance with respect to existing methods

Done.

e) Any future applications of the technique

Done.

13. 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.

Done.

14. Please include a scale bar for figure 3.

Done.

15. Please sort the materials table in alphabetical order.

Done.

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.

Done. The only figure that is not shown in the video is the sub-optimal cryo-EM grid atlas due to time constraints in the video.

2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol in imperative tense.

In accordance with JoVE guidelines, we have strived to make the narration as close to the written protocol as possible; however, for brevity and flow we have omitted some details that are best described in the protocol.

3. Please ensure that the protocol subheadings are the same in the video and in the text.

Done

4. Title Cards:

- 02:29 Consider using the same style of chapter title cards in every section of the video

- 02:54 Consider using the same style of chapter title cards in every section of the video.

The cards at 2:29 and 2:54 are subheadings and not title cards. In order to emphasize to the audience that these sections are subheadings, rather than distinct chapters, we elected to use a different style. The white background with black text directly corresponds to headings in the text while the white text on a black background represents subheadings. Each chapter title card and subheading title card directly correspond to the written text.

- 07:31 - 08:35 Please place your text overlay in a black space i.e the left corner, so it can be more properly visible to the audience.

As requested, we have moved the text to the upper left corner of the video and added a semi-transparent dark gray background to the text for improved visibility.

5. Video Editing Content:

- There doesn't appear to be a Conclusion. Please add a Conclusion section to the video

Thank you for catching this error on our part. We have added a conclusion section, from 7:29 to 9:10, with an appropriate title card. This section shows the full workflow without interruption and results that are obtainable with manual plunge freezing. We have also added narration to this section of the video to emphasize key points and make it more consistent with the rest of the video.

6. Audio Editing and Pacing:

- Audio levels are not balanced properly. Please ensure audio level peaks average around -9 dB.
- 8:49 Audio Levels are quite low. Please ensure audio level peaks average around -9 dB.
- 07:29 - 08:44 There seems to be a lack of audio. Please add the narration to this section of the video.

The audio levels on all tracks have been adjusted to ensure the audio level peaks are around ~9 dB. As stated above, we have modified this section and have now included narration to emphasize key points as well as making the narration more consistent with the rest of the video.

Once done, please ensure that the video is no more than 15 min in length. Please upload the video to <https://www.dropbox.com/request/Nui6vhhINxDvLUEPOFis?oref=e>

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors describe an optimized workflow to manually plunge cryo-EM samples. This is a tricky technique that is not easily taught, therefore this submission is of great utility to the cryo-EM community

The video is great!

Major Concerns:

None.

Minor Concerns:

Could authors advise on what to do when EH&S does not allow ethane/liquid nitrogen in the cold room?

We thank the reviewer for their comments and are glad they enjoyed the video.

We have added the following language to section “4.2.1” within the manuscript.

“For situations in which liquid nitrogen cannot be used in a cold room, we recommend plunge freezing in a cool and well-ventilated space.”

Reviewer #2:

Manuscript Summary:

The authors nicely described a detailed protocol for preparing the cryogenic electron microscopy specimen that is critical to get high-resolution structures. Cryogenic electron microscopy and single-particle analysis have become popular such that non-experts in this field can use this method for their research. The manual blot-and-plunge freezing device in this paper has great potentials. Compared to other commercially available sample preparation devices such as Gatan CP3, Vitrobot, or Leica GP2, the manual plunger described here is cost-effective and very flexible in many ways. The authors provided a great alternative to the commercial devices and shared the critical tips for succeeding in cryo-EM grids preparation. I enjoy reading the manuscript and highly recommend publishing their work.

Major Concerns:

The only primary concern is the lack of detailed descriptions about the plunger system and the 3D printed parts, such as the rotational grid storage platform, the dewar grid base, and ethane platform, etc. Can the author provide those 3D print design files too?

We thank reviewer 2 for their time reviewing this manuscript. For the descriptions and designs of the plunger system (e.g., plunging arm and 3D-printed dewar, etc.), we have added a new citation #53 that contains the description and files for the system.

Minor Concerns:

Excel file in the PDF. Some column width is too small to include all the characters.

Thank you for catching this error. We have substituted the links with information about those products.

In the excel

Item 13

Ethane propane tank. It is not clear what it is.

We have clarified this in the Excel file.

A mixture of ethane and propane in a high-pressure tank? The link is only to the website. Could the author provide a more specific description and a direct link to the item?

Done

Item 14

Ethane tank. Not clear either.

By the way, what kind of purity does the author recommend for ethane or propane gas?

We have clarified this in the Excel file.

Manual plunger stand (black stand + foot pedal)

More specific descriptions and links are needed, and why BioRxiv is in the company column? Is this a link to another bioRxiv paper?

What is Mark 5? Are the authors referring to the Vitrobot Mk IV?

They are essential items for other labs to reproduce the protocol in this paper.

We have added additional clarifications to these sections of the manuscript and have provided a new citation that further details these components and how to acquire them.

Item 34

The link is no longer working. Maybe it is not crucial as the plunger stand and Mark 5 plunging platform. But it would be better if the author specifies what Tygon tubing is used for and if there is any specific requirement.

Tygon tubing is used to connect the output of the ethane gas regulator to the metal dispensing tip. The length of Tygon tubing required depends on each lab setup but should be long enough to allow free movement of ethane tip and wide enough to fit the valves. We have included this language in section 4.4 of the protocol.

A few essential items are missing.

Dewar grid base. Is this commercially available, or is it 3D-printed? Can the author provide a 3D print design file? And recommended material for 3D printing?

Is the ethane platform on top of the grid base also 3D printed?

The brass ethane vessel. It would be better if dimension and catalog number were provided.

The foot pedal is part of the manual plunger system?

Key components of the plunger system can be sourced using parts in the Excel file and a new citation that contains the description and files for the system (reference #53) contains necessary information pertaining to the 3D printing and assembly. We hope the audience finds this information sufficient.

Reviewer #3:

Manuscript Summary:

Nguyen et al describes a detailed protocol on sample preparation for single-particle cryo-EM. Although the vitrified sample for cryo-EM imaging can be prepared in an automatic manner, a manual plunge freezer provides an alternative cheaper option and allows for optimizing different conditions or accommodation for different setups, such as light activation. The authors provide an extensive review and a detailed protocol in using the blot-and-plunge technique for a manual plunger. However, there are some confusing statements that seemed to diminish the advantage of the technique. Some issues of this technique will also need to be addressed in the manuscript.

Major Concerns:

1. In the Introduction (line 64), authors stated that "a skilled researcher can achieve the same, if not better, reproducibility as the robotics mentioned above." Does this mean that the manual blot-and-plunge technique has a concern about the procedure reproducibility? Or does this mean this technique only allow for researchers with any specific skills?

We thank the reviewer for their careful reading of our manuscript, we have addressed the reviewers concern by updating the language used within the manuscript to "Indeed, a manually-operated blot-and-plunge device can achieve high-quality cryo-EM grids at a fraction of the cost of robotic counterparts." We feel this better reflects the reproducibility and accessibility of the method proposed here.

2. The safety concerns of using liquid ethane should be addressed in which is applied in the procedure.

We thank the reviewer for catching this error. We have added additional comment to section 4.1 detailing the flammable and cryogen hazards.

3. The experimental parameters for glow discharging on the EM grid are not provided.

Due to the large diversity in grids now available to researchers, the experimental parameters for glow discharging will vary drastically from grid type to grid type. To accommodate this variability, we opted to not include experimental parameters for glow discharging.

4. Air-water interface problem has been extensively addressed in the cryo-EM field. Authors should discuss what could be the advantage or disadvantage of using the manual blot-and-plunge technique and what could be improved to minimize this problem.

While we agree with the reviewer that the air-water interface poses a serious problem for the cryo-EM community, we feel that it is beyond the scope of this paper as we lack sufficient evidence to argue one way or another. Rather, we focused on how this protocol could be adapted to change ice thickness parameters and particle distribution

that can be combined with other small molecule additives to circumvent the air-water interface. We have added reference #25 to the introduction for their perusal.

Minor Concerns:

1. Recommend labeling all the materials in the figure mentioned in the text, for example, the 'spinning grid disc'.

We have referenced this item in the legend of Figure 1 for clarity. Reference #53 added within the main text where the spinning grid disc is referenced provides additional information.

2. In Step 4.3, it is recommended to check whether the cooled brass ethane vessel is empty before adding cryogen into it.

In our experience, the addition of the ethane gas to the ethane vessel with liquid nitrogen present leads to immediate loss of the liquid nitrogen and does not pose a serious concern for the final liquid ethane quality.

3. In Step 5.3, authors recommended to use a Gatan Solarus II plasma cleaner to treat the grids before use. Authors should provide the reason and in what conditions this will be necessary.

In accordance with JoVE guidelines, we have removed any reference to specific products and have replaced this item with "glow discharger or plasma cleaner"

4. What kind of a blotting paper will be recommended to use? Does the quality of a blotting paper affect the results? This has not been described in the manuscript.

Information of this item has been listed in the Excel file

5. In Step 6.7, does the waiting time vary with the ambient humidity? Please advise.

In our experience the waiting time does not vary significantly but as referenced in the main protocol, further evaporation of the thin film once the blotting paper is removed leads to more evaporation in lower humidity. To this end, we recommend preparing grids in high humidity, if possible.

6. In Figure 2, please label the solid ethane ring in the figure.

Done.

7. In Figure 3A legend, a grid montage image does not show the ice thickness of a vitrified sample. Please revise.

In our experience, the amount of holes visible within a cryo-EM grid square is a first approximation of the thickness of the ice in the region, with regions of thicker ice locating to grid squares that are less visible. Of course, other metrics must be used to adequately measure the ice thickness.

8. In Figure 3B legend, what is the "aligned" micrograph? Does it mean the registered frame average of an electron movie?

We have changed “aligned” to “motion corrected” for clarity.

Reviewer #4:

Manuscript Summary:

Manuscript JoVE62765 entitled "Manual Blot-and-Plunge Freezing of Biological Specimens for Single-Particle Cryogenic-Electron Microscopy" describes the detailed steps of setting up and operating a manual plunge freezing device to produce high-quality cryo-EM grids with even ice thickness and sample distribution. The manuscript is very well-written and clear. It provides a wealth of important experimental details that will be helpful to both new and expert users. Publication is recommended upon consideration of several minor concerns.

Major Concerns:

N/A

Minor Concerns:

Line 35: I recommend the formal use of cryo-EM instead of the informal "scope."

Done

Line 38: Use "cryo-EM grid preparation" instead of "cryo-EM sample preparation."

Done

Line 46: "i.e., 3 μ L": My recommendation is to use "e.g." instead, or preferably a range such as 1-5 μ L as different volumes are used for plunge freezing.

Done

Line 60: "increased use" instead of "style."

Done

Lines 61-63: The sentence is misleading as most often the blotting time of different types of samples such as between different proteins for single particles needs to be optimized. The sentence implies that differences exist only between single particles and cells and not within these sample types/groups.

We thank the reviewer for catching this. We have removed this language and modified to indicate further that these steps needs to be optimized on a sample by sample basis, irrespective of sample type (e.g., protein, RNA, etc.).

Line 64: A word is missing after "reproducibility", which should read "reproducibility with a plunger."

We have clarified that this refers to ice thickness.

Line 65: A word is missing after "and": "manual plunge freezing device."

Corrected

Line 68: The reference is missing.

Corrected

Line 75: Use "reproducible" instead of "producible."

Corrected

Lines 88-91: I recommend combining Notes 1.2.1 and 1.2.2 as Note 1.2.1 provides the explanation for Note 1.2.2.

Done

Line 97: It would be helpful to include a note on the optimal length of the filter paper strips and whether a specific minimum or maximum length should be avoided.

Done

Line 107: A word, possibly "adjacent", is missing in the sentence.

Corrected

Line 201: Is "freezing" the right word, or would "condensing" be more appropriate? Based on the following text, solidification rather than condensation to obtain liquid ethane is the goal of this step. It would be helpful at this point to make the overall goal of the step clear, which is to obtain a solid for proper cooling of the ethane vessel before the ethane is liquefied.

Corrected

Line 246: Is the word "adjacent" missing after "dewar"?

Done

Line 304: A figure or text describing the degree of bending of the blotting paper would be helpful here.

Done