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Manual Blot-and-Plunge Freezing of Biological Specimens for Single-Particle Cryogenic Electron Microscopy

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TITLE:

Manual Blot-and-Plunge Freezing of Biological Specimens for Single-Particle Cryogenic Electron Microscopy

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SUMMARY:

This manuscript outlines the blot-and-plunge method to manually freeze biological specimens for single-particle cryogenic electron microscopy.

ABSTRACT:

Imaging biological specimens with electrons for high-resolution structure determination by single-particle cryogenic electron microscopy (cryoEM) requires a thin layer of vitreous ice containing the biomolecules of interest. Despite numerous technological advances in recent years that have propelled single-particle cryoEM to the forefront of structural biology, the methods by which specimens are vitrified for high-resolution imaging often remain the rate-limiting step. Although numerous recent efforts have provided means to overcome hurdles frequently encountered during specimen vitrification, including the development of novel sample supports and innovative vitrification instrumentation, the traditional manually operated plunger remains a staple in the cryoEM community due to the low cost to purchase and ease of operation. Here, we provide detailed methods for using a standard, guillotine-style manually operated blot-and-plunge device for the vitrification of biological specimens for high-resolution imaging by single-particle cryoEM. Additionally, commonly encountered issues and troubleshooting recommendations for when a standard preparation fails to yield a suitable specimen are also described.

INTRODUCTION:

Single-particle cryogenic electron microscopy (cryoEM) is a powerful structural technique that can be used to solve structures of dynamic biological specimens to near-atomic resolution[1–4]. Indeed, recent advances in direct electron detector technologies[4–10], improvements in electron sources[4,11–14], and electromagnetic lens stability[15], coupled with the continued development of data acquisition[16,17] and analysis software packages[18,19], have enabled researchers to now routinely determine structures of well-behaved specimens to 3 Å resolution or better[4,11,13,14,20–23]. Despite these improved imaging and data processing capabilities, cryoEM grid preparation

remains the largest impediment for successful high-resolution structure determination and often serves as a considerable bottleneck in the EM workflow[24–27].

CryoEM relies on the imaging of biological samples in aqueous solutions that are frozen to form a thin film of "glass-like" ice – a process known as vitrification – that preserves the native biochemical state. Vitrification of biological samples for cryoEM dates back over 40 years[28–30] and many techniques and equipment that have been developed for this process rely on the originally detailed blot-and-plunge method[31–35], whereby a small volume of sample (e.g., 1-5 µL) is applied to a specialized EM grid before the excess solution is removed using physical interaction of the grid with blotting paper. The timing of this process is usually empirically determined for each specimen as a critical component of freezing samples is the thickness of the vitreous ice film – if the ice is too thick then imaging quality deteriorates dramatically due to increased scattering of the electron beam while ice that is too thin can restrict protein orientations and/or exclude particles from the center of the grid foil holes[36]. This reliance on the perfect ice thickness for single-particle cryoEM has led to a wide array of techniques and equipment that can freeze samples, including robotics[37,38], microfluidics[42], and ultrasonic or spraying devices[27,39–44]. In recent years, some of the most popular sample preparation devices rely on the use of robotics for automated freezing of samples using the blot-and-plunge technique[45]. While these devices are designed to reproducibly create proper ice thickness for imaging, they often remain too expensive for individual labs to purchase and operate and are generally found within cryoEM facilities at hourly rates for usage. In recent years, the original manual blot-and-plunge technique has come back into increased use[3,47–52]. Indeed, a manually operated blot-and-plunge device can achieve high-quality cryoEM grids at a fraction of the cost of robotic counterparts, Furthermore, manual blotting also offers more users control over blotting as researchers can adjust the type of blotting (i.e. back-blotting of the grid, front blotting of the grid, etc.), and blotting time based on each individual sample and research questions.

In this article, we provide details on how to effectively freeze biological samples using a traditional manual blot-and-plunge vitrification device coupled with a custom-designed dewar platform[53]. Best practices, including preparation of the cryogen, grid handling, sample application, and blotting, as well as common pitfalls and recommendations on how to overcome these hurdles are provided. Advice on how to increase ice thickness reproducibility between grid preparations and how to modify sample blotting based on biological specimen type are discussed. Given the low cost associated with the purchase and operation of the manual plunger described in this manuscript, labs across the globe can prepare biological specimens for cryoEM in a cost-effective and reproducible manner.

PROTOCOL:

1. Prepare the manual plunging environment

NOTE: Estimated operating time: 5-30 minutes

1.1. Locate the manual plunger in a 4 ˚C cold room where a humidifier can be co-located to maintain the room close to 100% relative humidity (RH) (**Figure 1A**).

CAUTION: Please consult with the institution's Environmental Health and Safety guidelines for the safe location of the manual plunger and recommended operations.

1.2. Prior to grid preparation, turn on the humidifier in the cold room to ensure the RH of the cold room is ≥ 95%.

NOTE: Grid preparation in low humidity can result in dehydration of thin films, alteration of buffer components due to evaporation, and a decrease in grid-to-grid reproducibility[46]. It is not recommended to freeze grids at <80 % RH.

1.3. Ensure the temperature of the cold room is at 4 ˚C.

1.4. Locate the manual plunger away from strong air currents (i.e., away from air conditioning unit vents) as they can lead to turbulence near grids and/or prominent ice accumulation on cold surfaces.

2. Prepare plunging materials and accessories

NOTE: Estimated operating time: 1-5 minutes

2.1. Use clean scissors to cut blotting paper circles into 1-1.5 cm wide and ~9 cm long strips. Avoid touching the center of the blotting paper and discard the smaller end pieces. It is important to ensure that the blotting paper strips are dry, clean, and free of contaminants. Separate the strips and place them in a 100 mm Petri dish.

2.2. Place a 22x22 mm square glass coverslip into a separate 60 mm glass Petri dish. This coverslip-containing glass Petri dish will be used to store, transfer, and glow-discharge grids.

NOTE: It is recommended to use an air-duster can to remove any visible debris from the slide prior to adding grids. An anti-static gun can also be used to remove any static electricity that accumulates.

2.3. Assemble and label grid storage boxes.

2.4. Acquire 4 to 6 clean and dry clamping tweezers and locate them to the manual plunger. Visually inspect each tweezer prior to plunging to ensure they are not damaged and are free of contaminants.

3. Prepare the cryogen dewar and manual plunger

NOTE: Estimated operating time: 5-15 minutes

3.1. Install the platform base at the bottom of the plunging dewar. Place the ethane vessel dewar on top of the platform base, add the brass ethane vessel, and then install the spinning grid storage platform.

3.1.1. Once liquid nitrogen (LN_2) is added to the plunging dewar, the height of the platform base can no longer be adjusted. Ensure that the grid base is installed properly and is level to limit grid and/or tweezer damage due to improper plunger height.

3.2. Prior to freezing, check the manual plunger and all ancillary equipment to ensure they are functioning properly to limit sample and/or grid loss.

3.3. Prior to each freezing session, replace the tape on the manual plunger arm used to hold the tweezers. The high humidity of the room can deteriorate the tape adhesive and decrease the ability of the tape to hold the tweezers, increasing the likelihood of tweezer damage and/or grid loss.

3.4. Adjust the lamp(s) near the manual plunger to ensure there is sufficient light to monitor sample wicking and ensure grid transfer is easily visualized to prevent grid damage and/or loss (see step 3.7). Use a ring lamp directly behind the plunger to visualize liquid movement and a flexible arm task light near the dewar to illuminate the frozen grids.

3.5. Adjust the foot pedal tension to ensure the dewar plunging arm is securely retained in place while in the raised position and is fully released when the pedal is depressed. Perform several “dry” runs prior to sample application to ensure the plunger is working properly.

NOTE: Improper adjustment of the foot pedal tension will result in premature release of the plunging arm (i.e., tension is set too low) and grid loss or incomplete plunging of the grid into the ethane vessel (i.e., the tension is set too high).

3.6. Place the plunging dewar at the base of the manual plunger directly below the plunging arm and secure it in place. Attach a pair of tweezers to the plunging arm using the attached tape. While holding the manual plunging arm, depress the foot pedal to *carefully* lower the plunging arm and adjust the travel of the plunging arm to ensure the grid will locate within the middle of the ethane vessel.

3.7. Use the bump stop at the top of the plunging arm to determine the final position of the plunging arm when the foot pedal is fully depressed (**Figure 1B**). Adjust the bump stop height on the plunging arm to adjust the location of the grid in the ethane vessel (**Figure 1C**).

NOTE: Improperly setting the plunging arm height can lead to the grid and/or tweezer damage (e.g., plunging arm height is set too low) or inadequate vitrification (e.g., plunging arm height is set too high).

3.8. Locate the dewar outside the cold room and proceed to preparing liquid ethane (step 4).

4. Prepare the cryogen

NOTE: Estimated operating time: 10-30 minutes

4.1. Assess the ethane tank, regulator, tubing, and ethane dispensing tip for any signs of damage. Immediately report and rectify any signs of damage before proceeding.

CAUTION: Compressed ethane and ethane:propane gas mixes are flammable and can pose a serious threat to life and/or result in injury if improperly handled. Please consult an expert if unsure how to operate or handle compressed gas tanks. Please refer to the institution's Environmental Health and Safety guidelines when handling flammable compressed gases. In addition, liquified ethane is a powerful cryogen that can pose a serious threat to life and/or injury if not handled properly. Please refer to the institution's Environmental Health and Safety guidelines when handling cryogens.

4.2. Acquire sufficient LN_2 in an appropriate LN_2 handling dewar (i.e., 3-4 L is typical for grid preparation and storage).

CAUTION: LN_2 is a cryogen that can pose a serious threat to life and/or injury if not handled properly.

4.2.1. Ensure all personal protective equipment are utilized to minimize the risk of injury. The vapor of LN_2 is an asphyxiant and should be handled in well-ventilated areas. Please consult an expert if unsure how to operate or handle cryogenic vessels and cryogens. Please refer to the institution's Environmental Health and Safety guidelines when handling cryogens. For situations in which liquid nitrogen cannot be used in a cold room, we recommend plunge freezing in a cool and well-ventilated space.

4.3. Outside of the cold room, cool down the plunging dewar by pouring LN_2 directly into the brass ethane vessel until the liquid nitrogen level reaches the top of the ethane vessel (i.e., just above the platform). Top off the LN_2 outside the ethane vessel as needed. Proceed to the next step when the LN_2 stops bubbling violently (approximately 5 minutes).

4.3.1. Add LN_2 directly to the brass ethane vessel to sufficiently cool the vessel prior to condensing ethane gas. Failure to properly cool the brass ethane vessel will dramatically increase the time it takes to condense ethane.

4.3.2. Once the dewar has reached LN_2 temperature, avoid overfilling the dewar such that LN_2 spills into the ethane vessel.

4.4. Ethane comes as a compressed gas and needs to be liquefied for use. The ethane tank utilizes a high-purity dual-stage regulator to control the gas flow. Connect tubing to the regulator

outlet valve and use a 14-gauge flat metal dispensing tip connected at the end for dispensing ethane.

4.5. Prior to opening the ethane main tank valve, make sure the pressure adjusting knob and outlet valve are closed all the way. Fully open the main tank valve and then open the outlet valve to ~50%. Slowly open the pressure adjusting knob until a slow gas flow is observed. Use the outlet valve to fine-tune the gas flow.

CAUTION: Always point the metal dispensing tip away from self when opening valves or making gas flow adjustments.

4.6. Slowly start the gas flow and assess the flow rate by inserting the tip of the ethane gas line into a small beaker of deionized water. Adjust the gas flow until the flow rate *moderately* disturbs the water.

4.6.1. Adjust the gas flow rate to ensure proper ethane condensing occurs – too slow of a flow rate will cause the ethane gas to solidify in the dispensing tip and too fast of a flow rate will result in intense bubbling and prevent freezing.

4.7. Prior to inserting the tip of the ethane gas line into the brass ethane vessel, clean and wipe the dispensing tip with delicate task wipes to remove any water.

4.8. In a smooth and quick motion, locate the gas dispensing tip at the bottom of the brass ethane vessel and begin moving the dispensing tip in a slow circle around the bottom of the ethane vessel. Solid ethane will form immediately but will quickly liquefy as more ethane gas is added/condensed.

4.8.1. Continue to move the metal ethane dispensing tip around at the bottom of the ethane vessel to liquefy the solid ethane. Fill up the ethane vessel to ¾ full of liquid ethane (2-3 threads from the top). Stop ethane gas flow by carefully removing the metal ethane dispensing tip from the brass ethane vessel and closing the outlet valve.

4.9. Top off the plunging dewar with LN_2, pouring gently on the side of the dewar to avoid LN_2 addition to the brass ethane vessel, until the liquid level just touches the brass ethane vessel. Place the foam lid on the plunging dewar to facilitate ethane solidification.

4.9.1. LN_2 must directly contact the brass ethane vessel to aid in the solidification of ethane. After approximately 5 minutes, the ethane within the brass vessel will become completely frozen solid. Add more LN_2 until it just touches the ethane vessel and proceed to the next step.

4.10. If the ethane has not frozen solid, then the brass ethane vessel is not cold enough for the remaining steps. Add more LN_2 until it just touches the ethane vessel and wait an additional 5 minutes. Ensure that the ethane within the brass vessel is completely frozen solid.

4.11. Open the gas outlet valve to produce an ethane gas flow at a similar rate as determined in step 4.6. Vertically place the metal ethane dispensing tip into the solid ethane and continue to move the ethane dispensing tip in a circular motion to melt the solid ethane.

4.11.1. Continue to add ethane until it is level with the top of the brass ethane vessel. Slowly remove the tip from the ethane vessel and close the ethane tank outlet valve. Cover the dewar with the lid for ~1-4 minute(s) to let the ethane solidify around the edges of the brass ethane vessel.

NOTE: An ideal ethane vessel will have a 2-3 mm symmetric ring of solid ethane at the perimeter of the brass ethane vessel with liquid ethane in the center (**Figure 1D** and **Figure 2**).

4.11.2. Ensure that the ethane be as cold as possible without solidifying to ensure proper vitrification of the biological specimen. Failure to properly prepare the liquid ethane can result in inadequate vitrification of the specimen, ice accumulation, and/or loss of specimen, each contributing to the deterioration of specimen quality for imaging.

4.11.3. If a solid ring of ethane does not form after 2-3 minutes, add more LN_2 to the dewar and cover for 2-5 more minutes.

4.11.4. If the ethane is solidifying too quickly, then use a large pair of clean tweezers to gently warm the ethane vessel and/or solid ethane to prevent complete ethane solidification. Once the ethane and LN_2 are stable, close all the ethane tank valves and locate the plunging dewar to the manual plunger. Secure the plunging dewar to the manual plunger.

CAUTION: Be extra careful when transferring the dewar as LN_2 can spill into the ethane vessel and solidify the liquid ethane. If need be, a clean set of tweezers can be used to melt any solid ethane in the middle of the vessel.

4.12. Test the location of the ethane dewar with a pair of empty tweezers to ensure the tweezer tip locates at the center of the ethane vessel and there is sufficient space for the grid and tweezer tip inside the liquid ethane (**Figure 1D**).

4.12.1. If the solid ethane ring is too thick for easy grid handling, then use a pair of room temperature, clean tweezers to melt the solid ethane and create more freezing area at the center of the ethane vessel.

5. Prepare EM grids

NOTE: Estimated operating time: 1-5 minutes

5.1. Add the grid storage box(es) to the dewar, unscrew the grid storage box lid(s), and make sure each lid can freely rotate to a new grid slot.

5.2. Carefully transfer grids from the grid storage box to the edge of the square glass coverslip with ~30-40% of the grid off the slide edge. Ensure that the grid foil is facing up. Ensure grids are covered when not in use. Placing the grids over the edge of the square glass coverslip provides ease of grid handling and decreases the chance of bending or damaging the grid during transfer.

NOTE: 4-6 grids are typically prepared at a time.

5.3. Render grids hydrophilic using a glow discharger or plasma cleaner.

NOTE: Please refer to the recommended guidelines for grid cleaning provided by the glow discharger/plasma cleaner manufacturer.

5.3.1. Use the grids within 10 minutes of plasma cleaning as the grids lose hydrophilicity and grid-to-grid reproducibility decreases after this time.

6. Prepare cryoEM specimen by plunge freezing

NOTE: Estimated operating time: >10 minutes (~1-3 min per grid)

6.1. Use a clean and dry clamping tweezer to pick up a cleaned grid, slide the plastic clamp down to fix the grid in place, and gently tap the tweezers to ensure the grid is properly secured.

6.1.1. Handle grids by the outer ring to prevent damage to the grid foil.

6.2. Apply 1 to 5 µL of sample to the prepared side (e.g., front or foil side) of the grid.

NOTE: The optimal volume and blotting time depends on the sample and needs to be optimized for each sample; larger volumes and more viscous samples require longer blotting times.

6.3. Secure the tweezer-grid-sample assembly to the manual plunging arm by wrapping tape around the tweezer handle.

6.3.1. Face the sample towards the user for traditional front blotting. If the sample requires back blotting, then locate the sample away from the user.

6.4. Hold a clean, dry, cut piece of blotting paper between the thumb and index finger of each hand.

6.4.1. Only handle the blotting papers from the edges and never touch the center as oils and other contaminants from hands/gloves can alter grid quality.

6.5. Rest hands on the edge of the plunging dewar to establish a stable position. Locate the blotting paper parallel to the grid surface approximately 1 cm away from the grid surface.

6.5.1. Use the middle section of the blotting paper to allow for complete fluid mobility and even wicking across the grid surface.

6.6. Gently slide and rotate the thumb and ring fingers towards each other to bend blotting paper toward the grid to initiate blotting. Maintain contact between the blotting paper and the grid surface during the entire blotting process.

6.6.1. Directly contact the blotting paper to the grid surface and maintain consistent contact across the grid surface.

6.6.2. Gently bending the blotting paper decreases bending of the grid and/or damage to the grid surface, and results in more consistent ice across the grid (**Figure 3A**).

6.7. Observe the mobile liquid front and once it stops progressing into the blotting paper begin counting for 4 to 6 seconds.

NOTE: Counting can occur once the blotting paper contacts the grid surface but lower grid-to-grid reproducibility can occur. The total blot time will depend on the grid type, foil type, sample concentration, and sample type (e.g., soluble versus membrane versus filamentous proteins). For more viscous samples longer blot times (e.g.., 5 to 7 seconds) will be required.

6.7.1. Important: Develop a reliable and consistent counting scheme to greatly enhance reproducibility during the freezing process.

6.8. Move the left, right thumb and index fingers in opposite directions to remove the blotting paper in a “snapping motion” away from the grid surface. Immediately depress the foot pedal to release the plunging arm and plunge the grid into liquid ethane.

6.8.1. Simultaneously remove the blotting paper and press the foot pedal to plunge the grid into the ethane as soon as possible for best freezing results. The longer the time between blotting paper removal and plunging, the more evaporation of thin films will occur and decrease grid-to-grid reproducibility.

6.9. Use one hand to stabilize the clamping tweezers, unwind tape carefully from around the tweezers and manual plunging arm.

6.9.1. Always maintain contact with the tweezer to prevent movement of the tweezer-grid and limit grid damage that occur from knocking the grid against the solid ethane.

6.10. Once clamping tweezers are free from the manual plunging arm, maintain the tweezer in one hand resting on top of the plunging dewar, ensure the grid remains in the liquid ethane. Carefully slide the plastic clamp away from the grid so the grid can be transferred. Maintain pressure on the tweezers to retain the grid.

6.10.1. With one swift motion, quickly transfer the grid from the ethane vessel into the LN_2 reservoir. Carefully place the grid in the grid storage box.

NOTE: Some ethane may solidify on the grid surface. Opening the tweezer slightly will break the ethane and allow for the grid to be dropped into the grid box.

6.11. Wrap the tip of the tweezers in a delicate task wipe to prevent frost accumulation. Set aside until the tweezers have returned to room temperature.

6.11.1. Have 4 to 6 tweezers on hand for ease of use. Each tweezer will be used for sample freezing and warmed before subsequent use.

6.12. Repeat steps 6.1-6.11 for each grid.

6.13. Once freezing has culminated, securely close the grid box and transfer to a proper storage location.

6.14. Carefully dispose of the liquid ethane and LN_2 and store all materials in a dry location.

REPRESENTATIVE RESULTS:

Successful execution of the blot-and-plunge protocol described here will result in a thin, uniform layer of vitreous ice that is free of any hexagonal ice, contaminants, and large gradients of unusable ice which can be observed under the electron microscope (**Figure 3**). Inconsistent contact of the blotting paper with the grid surface, prematurely removing the blotting paper, or moving the blotting paper during grid contact can decrease the quality of the vitreous ice and lead to inconsistent ice thickness across the EM grid (**Figure 4**)

FIGURE AND LEGENDS:

Figure 1: Specimen plunging room and required equipment. A) Staged cold room for the manual freezing of biological specimens using a traditional blot-and-plunge device outlined in this article. Necessary equipment is shown and labeled accordingly. **B**) To adjust the working height of the manual plunger, adjust the bump stop by sliding it up and down the manual plunging arm and securing it by tightening the screw. **C**) Zoomed-in view of the ethane vessel and spinning grid storage platform to indicate proper height and location of the clamping tweezers and grid inside the empty brass ethane vessel. The tweezers and grid should not contact the sides or bottom of the brass ethane to avoid damage. **D**) Proper height and location of the clamping tweezers and grid in liquid ethane. The tweezers and grid should enter the liquid ethane in the center, avoiding contact with the solid ethane at the perimeter.

Figure 2: Prepared liquid ethane. Zoomed-in view of the plunging dewar showing the state of liquid ethane in the brass ethane vessel prior to specimen freezing. The 2-3 mm ring of solid ethane within the brass ethane vessel is clearly visible.

Figure 3: Representative apoferritin images obtained using the manual blot-and-plunge technique. (**A**) Representative atlas of a cryoEM grid showing the ice thickness and quality of grid squares that can be obtained using the manual blot-and-plunge technique. (**B**) Motion-corrected micrograph of vitrified mouse apoferritin acquired using a 200 kV transmission electron microscope equipped with a direct electron detector at the University of California, San Diego's CryoEM Facility.

Figure 4: **Representative atlas of a sub-optimal cryoEM grid showing inconsistent ice thickness across the grid, numerous broken squares, and areas in which the ice is too thick to image the specimen.**

DISCUSSION:

The vitrification of biological specimens for imaging by single-particle cryogenic electron microscopy (cryoEM) remains a critically important step for successful structure determination. The manual blot-and-plunge method described in this protocol represents a cost-effective, reliable, and robust method for quickly freezing biological samples in thin films of vitreous ice for cryoEM imaging. Using the methods outlined in the manuscript, researchers will be able to assemble and operate the manual plunger, prepare cryogen suitable for flash-freezing biological samples, and manually blot-and-plunge EM grids containing biological specimens. While this method is quite robust, care should be taken during critical steps in this procedure to obtain optimal ice thickness and quality for high-resolution imaging. We have outlined several of these critical steps below and provide recommendations on how to troubleshoot these steps.

It is imperative to properly position the manual plunging arm to ensure that the grid locates at the center of the liquid ethane within the brass vessel after plunging. Improper height or position of the plunging arm and/or not securing the tweezers properly will lead to damage to the clamping tweezers, the EM grid, and possibly the manual plunger. As discussed above, we always perform at least one trial run prior to preparing biological specimens to verify that the EM grid will locate to the center of the brass ethane vessel after successful plunging (**Figure 1C**). In addition, we also make minor adjustments to the location of the plunging dewar after each grid freezing to fine-tune grid placement within the ethane vessel (**Figure 1D**).

The proper preparation of the ethane cryogen is critical for obtaining thin films of biological specimens in vitreous ice. We have observed that the presence of a 2-3 mm ring of solid ethane around the inner edge of the brass ethane vessel ensures that the temperature of the liquid ethane is optimal for sample vitrification (**Figure 2**). Indeed, after each grid has been frozen, we monitor the quality of the ethane and make minor adjustments – slightly warming the vessel if too much ethane has solidified or cooling the ethane if the system has warmed up – as needed. We have found that the edge of a room temperature tweezer is sufficient to liquefy solid ethane while covering the dewar with the foam lid for 1-5 min is enough time to allow the ethane to cool. Importantly, we make these adjustments prior to preparing the grid surface (i.e., plasma cleaning) and applying the sample to the grid as this can introduce another variable to grid preparation that is not reproducible.

Finally, we recommend developing a standardized blot-and-plunge routine – sample application, sample blotting, and blotting time – to increase grid-to-grid reproducibility. Bending the blotting paper towards the EM grid allows for uniform contact of the paper with the grid and produces more consistent ice thickness across the entire grid, resulting in even particle distribution within the grid holes (**Figure 3A** and **Figure B**, respectively). This method of blotting is in contrast to robotic blotting devices that interact with the specimen at an angle that may result in a gradient of ice thickness across the grid. In addition, this bending of the blotting paper also decreases the chance of damaging the EM grid upon contact with the blotting paper by buffering the force being applied by the user. After the desired blotting time, quickly straighten the blotting paper by performing a snapping motion to rapidly move the blotting paper away from the grid surface before plunging to prevent damage to the grid upon release of the manual plunging arm. We have found this blotting method and the snapping motion of the blotting paper, when timed with the simultaneous release of the manual plunging arm via the foot pedal, limits evaporation of the thin film before vitrification and increases grid-to-grid reproducibility.

The manual blot-and-plunge method described here is a robust and reliable method that helps lessen some of the financial burden cryoEM can place on emerging labs. While this method is reproducible, creating high-quality vitreous ice that is suitable for cryoEM relies on the experience and skill of the individual researcher. Although robotic plungers and other emerging technologies automate several aspects of the freezing process, they are generally limited by how much control they offer to researchers and often incur a high price to purchase and operate. With the method outlined in this protocol, researchers will be able to utilize an affordable and versatile EM grid preparation platform that offers flexibility to optimize the plunging conditions (i.e., blotting paper types, blotting angle, blotting durations, blotting directions, etc.) based on sample types and characteristics.

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DISCLOSURES:

We have nothing to disclose.

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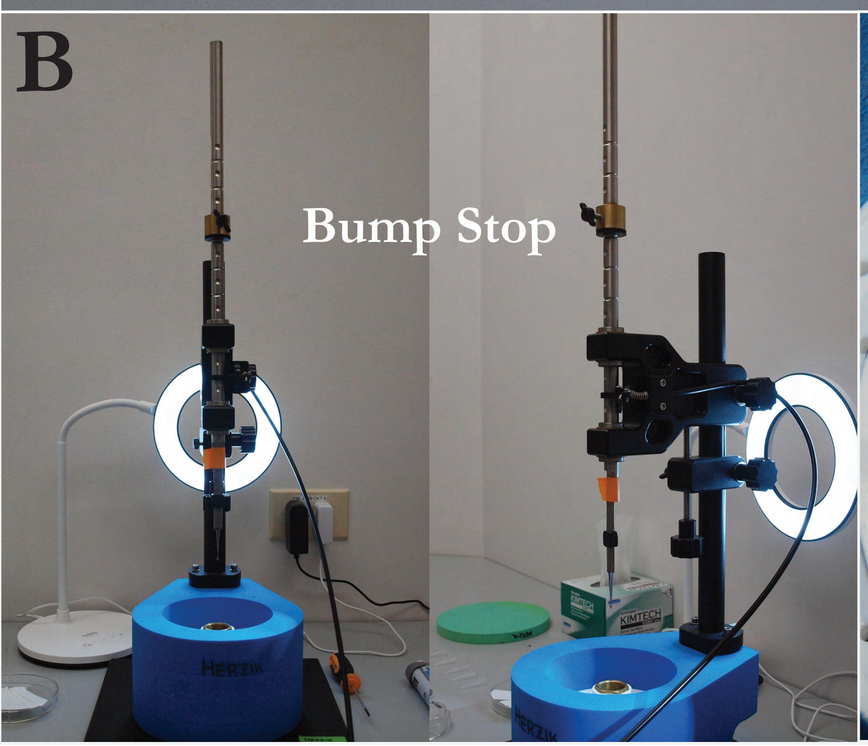
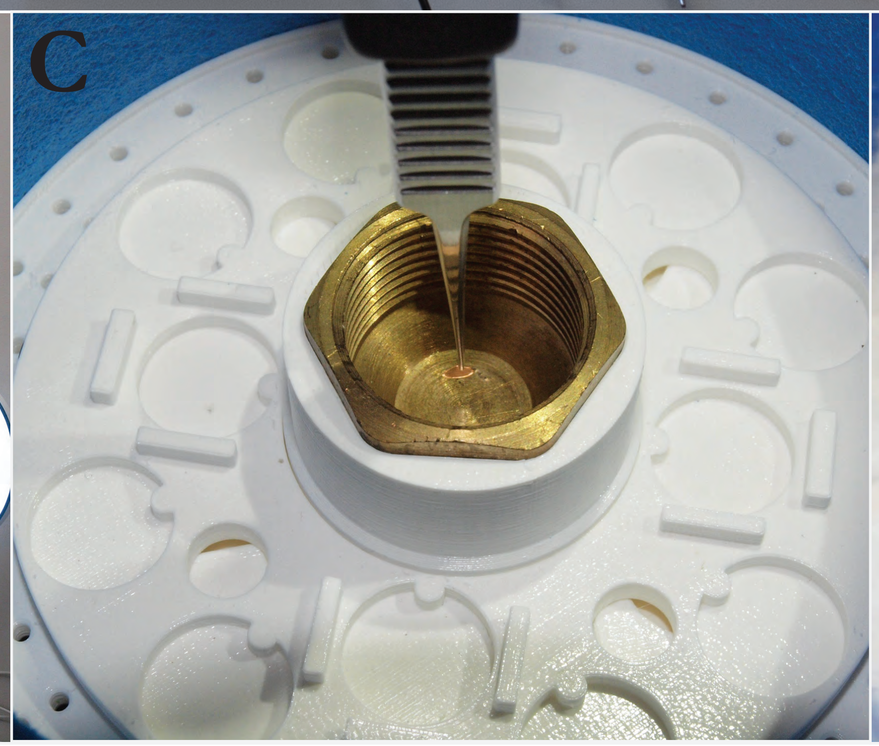
Figure1

Click here to access/download;Figure;Figure_1.sm.pdf





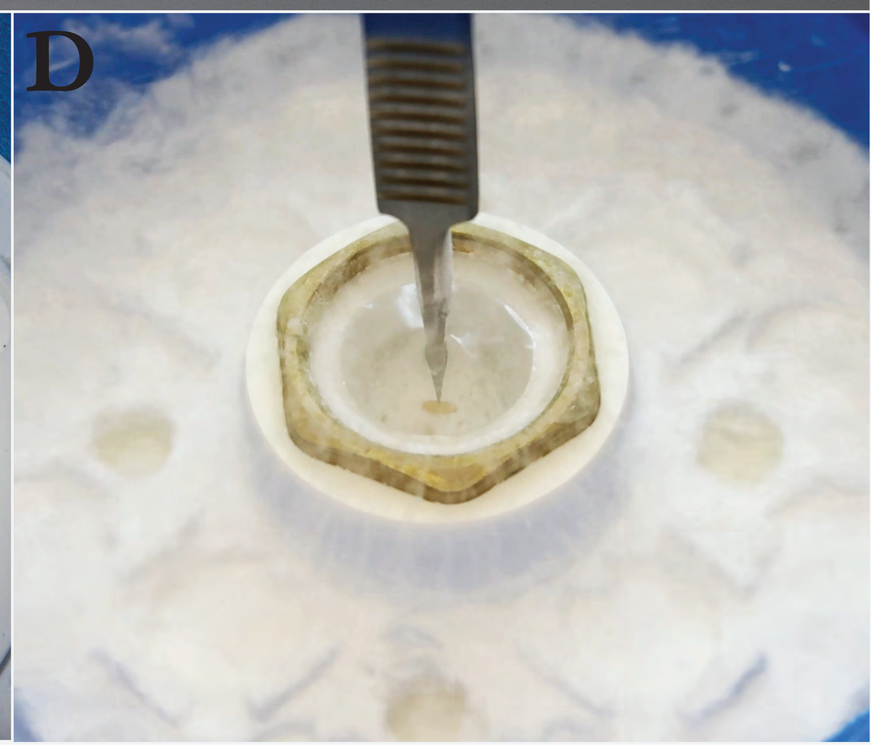


Figure2

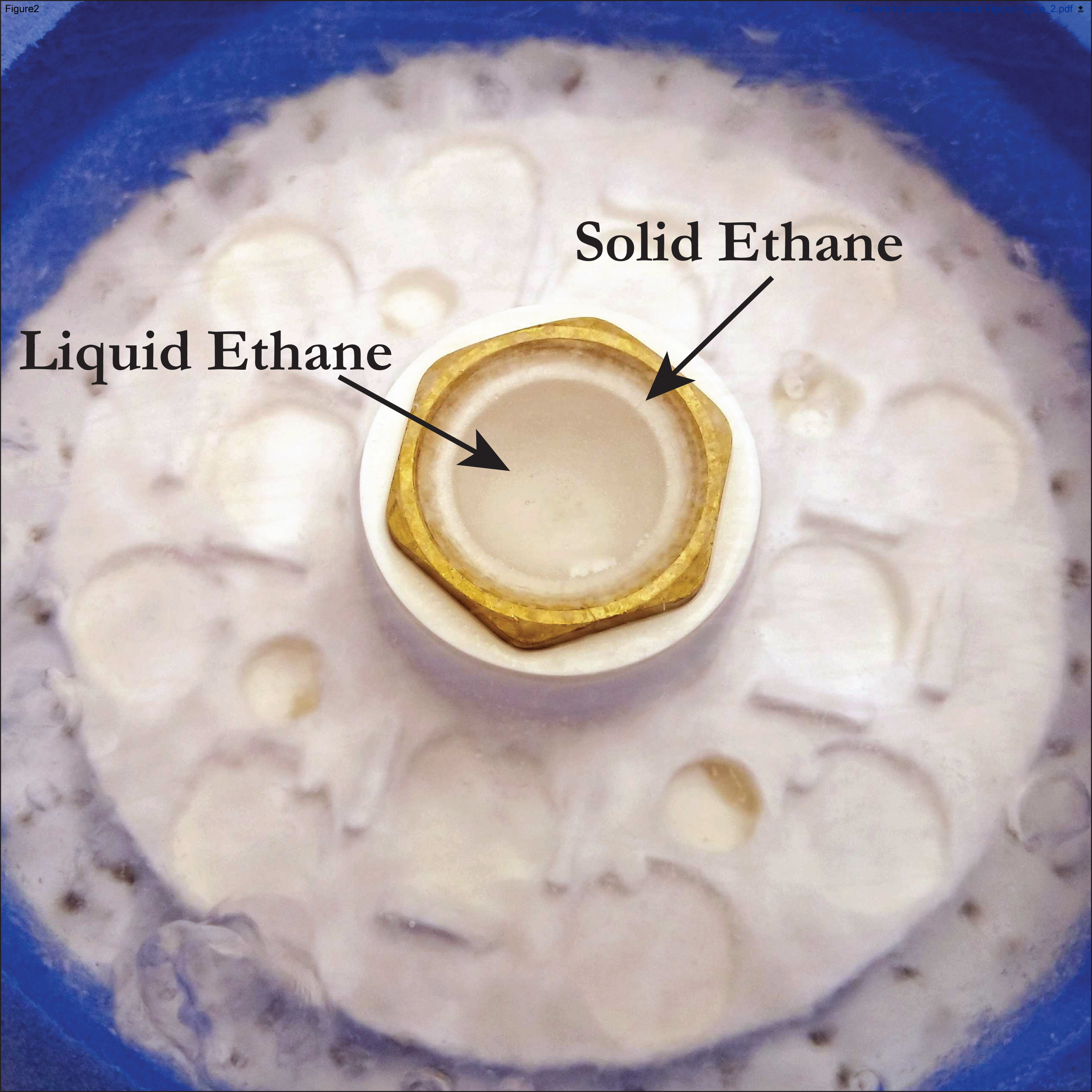


Figure3

Click here to access/download;Figure;Figure_3.pdf

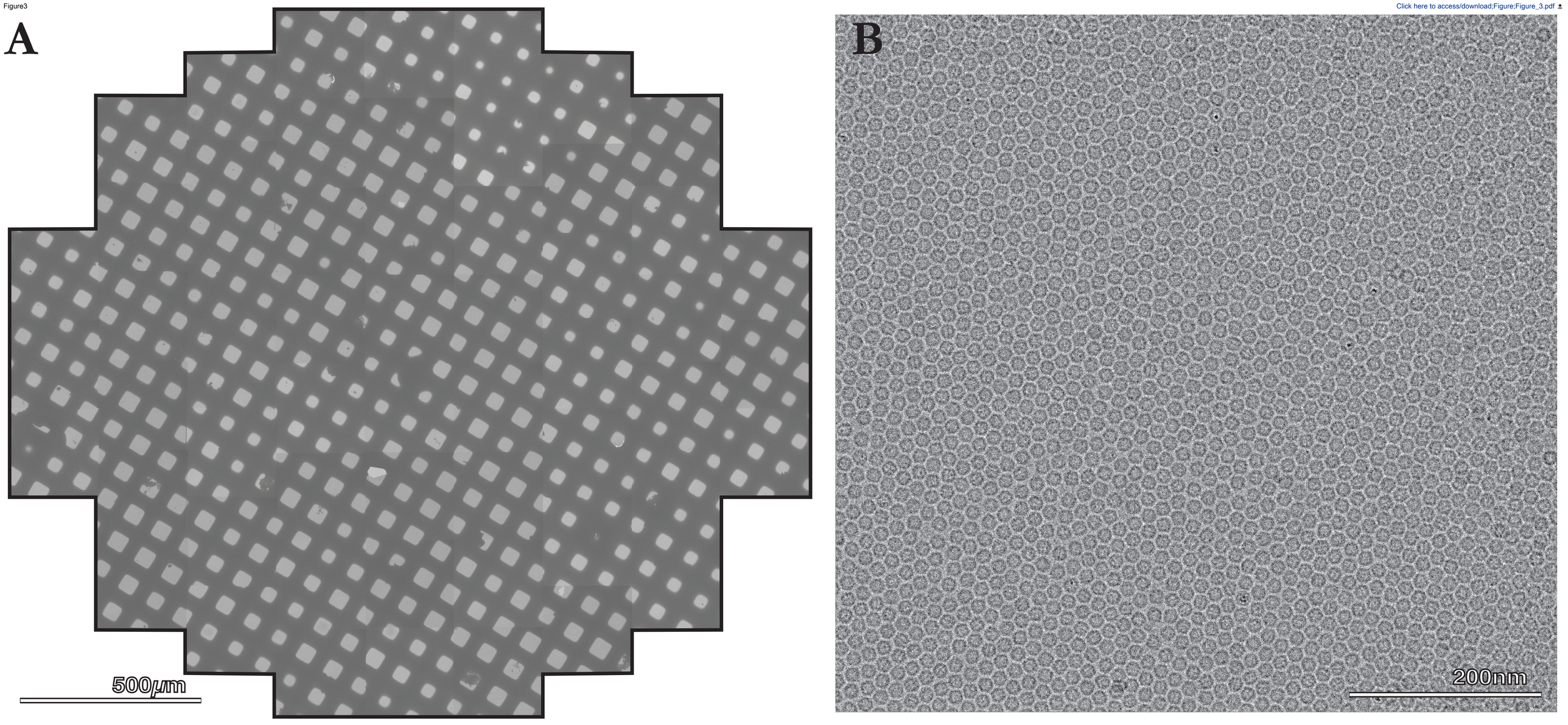
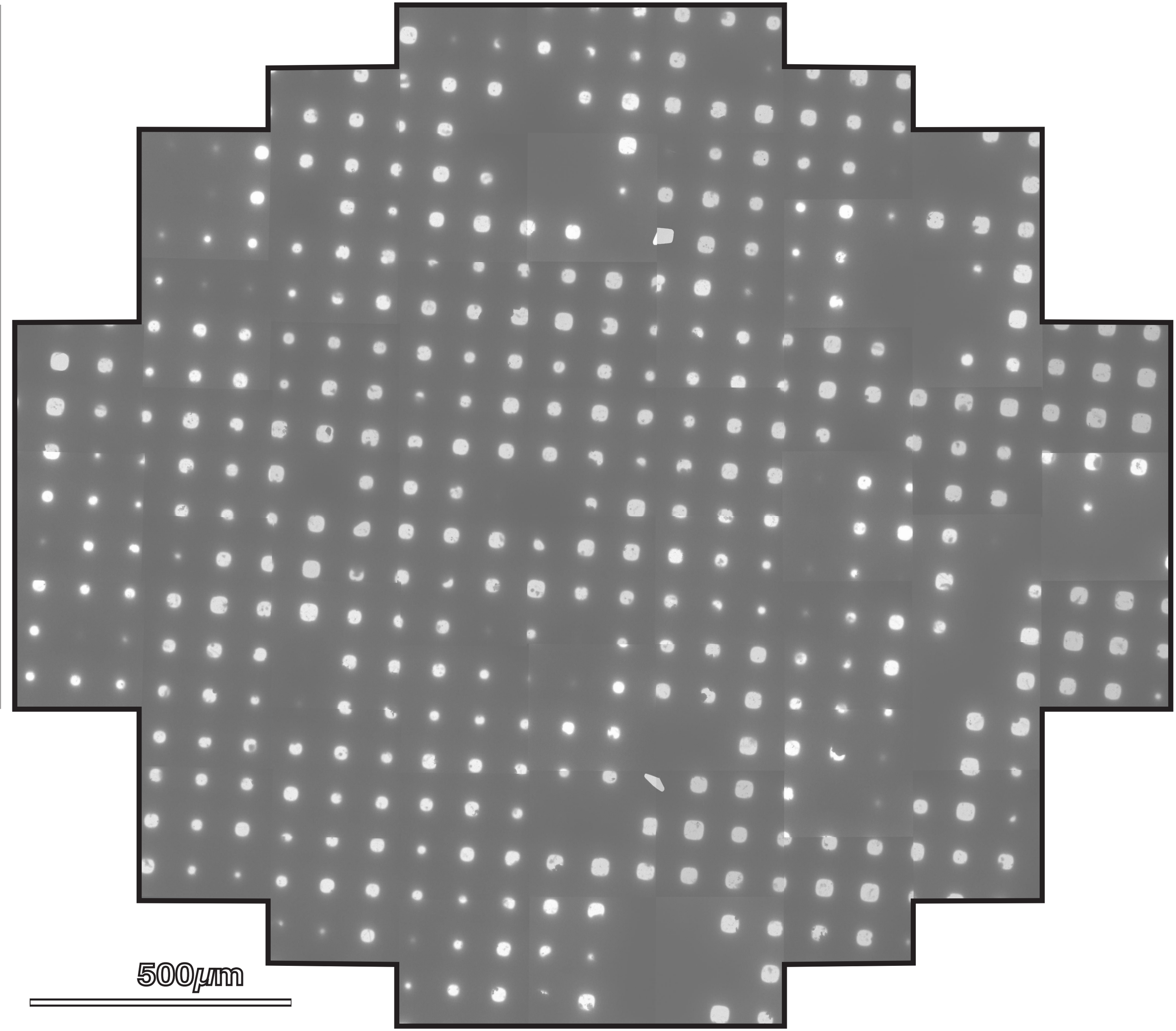


Figure4

Click here to access/download;Figure;Figure_4.pdf



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-ParticleCryogenic-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 µL": My recommendation is to use "e.g." instead, or preferably a range such as 1-5 µL 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

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 revise the following lines to avoid previously published text: 49-51, 52-53, 54-57, 61-65

Done. There should no longer be conflicting language.

Changes to be made by the Author(s) regarding the video:
1. Title Cards:
• 02:28 Please use same style chapter cards,i.e White Background and Black Text.

Done.

• 02:54 Please use same style chapter cards,i.e White Background and Black Text.

Done.

• Please use the same protocol subheaders as the written protocol. Alternatively, you can change the subheaders in the written protocol instead to match the headers in the protocol section of the video.

Done. We have removed/altered any subheaders in the video not present in the written protocol.

2. Video Editing Content:
• Please remove the conclusion section from here and add it after the Result section i.e, 09:12 . For more information see the JOVE video Guidelines.

Done.

3. Audio Editing and Pacing:
• Audio levels are low. Please increase the audio level and ensure that the audio level average around -9dB.

Done.