Video Article

Low-Cost Cryo-Light Microscopy Stage Fabrication for Correlated Light/Electron Microscopy

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Abstract

The coupling of cryo-light microscopy (cryo-LM) and cryo-electron microscopy (cryo-EM) poses a number of advantages for understanding cellular dynamics and ultrastructure. First, cells can be imaged in a near native environment for both techniques. Second, due to the vitrification process, samples are preserved by rapid physical immobilization rather than slow chemical fixation. Third, imaging the same sample with both cryo-LM and cryo-EM provides correlation of data from a single cell, rather than a comparison of "representative samples". While these benefits are well known from prior studies, the widespread use of correlative cryo-LM and cryo-EM remains limited due to the expense and complexity of buying or building a suitable cryogenic light microscopy stage. Here we demonstrate the assembly, and use of an inexpensive cryogenic stage that can be fabricated in any lab for less than \$40 with parts found at local hardware and grocery stores. This cryo-LM stage is designed for use with reflected light microscopes that are fitted with long working distance air objectives. For correlative cryo-LM and cryo-EM studies, we adapt the use of carbon coated standard 3-mm cryo-EM grids as specimen supports. After adsorbing the sample to the grid, previously established protocols for vitrifying the sample and transferring/handling the grid are followed to permit multi-technique imaging. As a result, this setup allows any laboratory with a reflected light microscope to have access to direct correlative imaging of frozen hydrated samples.

Video Link

The video component of this article can be found at <https://www.jove.com/video/2909/>

Protocol

1. Fabrication and Assembly

1. Place the aluminum heat sink inside the 22.96 cm (9") pie pan approximately 2 cm from the side of the pan, as shown in Figure 1c. Using a black marker pen, mark holes for the aluminum heat sink. **Note:** If the aluminum block (purchased at a local scrap metal shop or online at <http://www.onlinemetals.com>) does not come with pre-drilled holes, you will need to arrange to have holes drilled and countersunk prior to step 1 to secure the aluminum block to the pie pan. We used 5 holes to secure the block to the platform and 4 extra holes to allow nitrogen gas bubbles to escape from under the aluminum.
2. Place the cryo-grid box near the location of the aluminum heat sink and mark the notch and each side.
3. Drill pilot holes for each marked position using a #17 drill bit for the aluminum heat sink holes and a #35 drill bit for the cryo-grid box holes.
4. Mount the aluminum heat sink using 5 - #10, 1.9 cm (3/4") flat head slotted bolts and corresponding nuts as well as 15 - #10 washers. Place two washers beneath the aluminum block and the third on the backside of the pie pan for each hole. **Note:** Unsecured overhangs of the aluminum block near the viewing area can result in vibrations that limit imaging capabilities of the stage. Check to make sure all mounting bolts are firmly tightened.
5. Insert 3 - #4, 0.95 cm (3/8") round slotted bolts and corresponding nuts from the backside of pie pan to act as the cryo-grid box mount.
6. Tape 4 chopsticks together in pairs with one chopstick atop another. Tape each pair to opposite edges of the cake pan to act as spacers.
7. Wet the top and bottom surface of the cake and pie pans respectively with ddH₂O.
8. Fill the cake pan with two layers of insulating spray foam. Wetting each layer before going to the next step.
9. Press the pie pan into the foam.
10. Flip the pans upside down and press on the cake pan until the spacers contact the pie pan. Place any weighted object atop the cake pan and let sit overnight to cure.
11. With a serrated knife, cut away the excess dried insulating spray foam from the edge of the pans and remove the chopsticks.
12. Remove the cake pan from the pie pan. The pie pan with aluminum heat sink is now insulated and will be referred to as the "cryogenic stage" (Fig 1a).
13. Place a transparent plastic clipboard (any color and greater than 3 mm thick) over the top of the cryogenic stage. Mark with a black marker the location of the cryo-grid box mount by drawing a circle approximately 1.5 times the diameter of a single round cryo-grid box. Cut out hole with a 1.9 cm (3/4") hole saw. This clipboard will be referred to as the "loading screen" (Fig 1b).

14. Put a new transparent clipboard atop of the cryogenic stage and place on the microscope with the heat sink directly under the objective lenses. With a black marker, mark the path of the objectives as they rotate.
15. Cut along the marked line removing a half circle from the edge of the clipboard. This can be accomplished with a jigsaw or using a 7.62 cm (3") hole saw multiple times as shown in the video.
16. Finally cut a 1.9 cm (3/4") hole away from the half circle but still within the area of the dish. This will be the port for refilling liquid nitrogen (LN₂) if levels drop while imaging. This clipboard will be referred to as the "viewing screen" (Fig 1c).

2. Sample Preparation

1. Dilute log phase yeast cells grown in synthetic complete (SC) media to an appropriate concentration of 1×10^6 cells/mL in water.
2. 2 mL of the diluted yeast are pipetted onto a R2/1 400 mesh holey carbon coated copper cryo-EM grid (SPI Supplies, West Chester, PA) and allowed to adsorb for 15 seconds.
3. Blot away excess liquid from the grid surface by touching the back of the grid to a torn piece of soft cellulose tissue (Kimwipe) for 2 seconds.
4. If necessary, the grid can be washed with 3 μ L drops of water (1-3 times), and wicked away as in step 3 by blotting from the back.
5. Before the final wash, the sample is loaded into the pneumatic plunge freezer and the hanging grid is washed as in step 2.4. After blotting the grid with torn tissue paper (Kimwipe) to remove the majority of the water from the surface, the grid is plunged into LN₂ cooled liquid ethane and transferred to a cryo-grid box for storage ¹. **Note:** it is important to leave a layer of liquid as thin as possible on the surface to keep the sample hydrated. Improper blotting will either dry out the sample or leave ice that is opaque to the electron beam.

3. Cryo-Light Microscopy

1. Fill the cryogenic stage with LN₂ and quickly cover with the loading screen (plastic clipboard with single 1.9 cm (3/4") hole).
2. Once the metal reaches LN₂ temperature, transfer the cryo-grid box through the 1.9 cm (3/4") hole of the loading screen and into the transfer mount created by the 3 screws described in part A5. Unscrew the cryo-grid box from its holder and remove the holder for improved access. Keep the cryo-grid box holder under LN₂ in a separate dewar until step 3.10. The cryo-grid box should also be kept under LN₂ to prevent sample grid warming.
3. Refill LN₂ in the cryogenic stage to just below the top of the aluminum heat sink. **Note:** LN₂ levels must be periodically checked and refilled during the imaging process to ensure LN₂ is continuously in contact with the heat sink. In general, the refilling occurs every 10 minutes through the fill port in the viewing screen.
4. Pre-cool the tweezer tip in LN₂, then transfer your grid(s) onto the flat viewing surface of the aluminum heat sink with tweezers, using the 1.9 cm (3/4") hole of the loading screen.
5. Carefully move the cryo-stage to beneath the reflected light microscope's objective apertures.
6. Place the transparent viewing screen atop the loading screen. Then slowly remove the loading screen by sliding it underneath the viewing screen. **Note:** At this point, the sample is most vulnerable. All air currents around the microscope should be minimized, including: breathing, a nearby door opening, people walking past, etc. We suggest wearing a facemask or hanging a transparent face shield below the microscope eyepieces as a precautionary measure.
7. Rotate the objective lenses into the cold nitrogen gas environment of the cryo-stage and scan for the sample on the flat heat sink viewing area. Using standard bright field light microscopy techniques focus on the grid with a low magnification objective to find the center and take an overview image. Specifications regarding the light microscopy lenses used for this experiment are listed in the Experimental Materials section. **Note:** LN₂ does not come in contact with the objective lenses and we have yet to see degradation of images or damage to the lenses from the effects of cooling.
8. Focus in high magnification on an area of interest and acquire data with bright field, dark field, polarized light or fluorescence imaging. Be sure to record the location of the area of interest on the low magnification bright field image for future correlation. If all precautions listed above for refilling the LN₂ are followed, then the positive pressure from the evaporating nitrogen is sufficient to maintain a contamination free environment (independent of room humidity) for the duration of imaging.
9. Once finished, replace the loading screen by sliding it over the top of the viewing screen. Remove the viewing screen by slowly sliding it beneath the loading screen.
10. Remove the cryo stage from the microscope and with pre-cooled tweezers, transfer the grid back to the cryo-grid box. Screw the cryo-grid box holder into cryo-grid box to seal against the environment and transfer the holder to a LN₂ dewar for storage and future imaging.

4. Cryo-Electron Microscopy

1. Following standard and established techniques, load the sample grid into a cryo-EM transfer holder while keeping the specimen at liquid nitrogen temperature at all times.
2. Insert the cryo-holder with sample grid into a Transmission Electron Microscope.
3. In a suitable low magnification view (50-500x nominal magnification), find the center of the sample grid.
4. Use the previously collected low magnification cryo-LM data from step 3.7 to determine the relative orientation of the grid's central copper tabs (as described in Figure 2) and identify exact areas of interest for correlation.
5. Proceed with microscope alignment, low-dose cryo-EM imaging and data collection.

5. Representative Results

The cryogenic stage (Fig 1a) is an effective way to gather cryo-LM data for cryo-fluorescence microscopy and correlative cryo-LM/cryo-EM analysis. Figure 2 shows how the combination of low and high magnification cryo-LM images allows you to build reference maps that direct you to specific areas in cryo-EM. The resulting cryo-LM reference map (Fig 2b) was utilized during cryo-EM data acquisition to locate exact regions shown in Figure 3.

Figure 1. Cryogenic Stage. a) The layout of the cryogenic stage consists of a cryo-grid box transfer mount indicated with a white arrow and an aluminum heat sink. Grids are transferred under IN_2 from the cryo-grid box and placed directly on the heat sink's viewing area as indicated by the black arrow. b) Image depicting the cryogenic stage with loading screen. The hole in the loading screen is placed directly over the cryo-grid box mount and acts as a port to transfer samples and for moving samples from the cryo-grid box to the sample viewing area on the heat sink with tweezers. c) The cryogenic stage in position under the objective lenses with the viewing screen in place. The special cutout on the viewing screen allows the objective lenses to easily swing into position without moving the cryogenic stage. The hole in the viewing screen away from the sample area acts as a IN_2 fill port to replenish IN_2 levels if necessary.

Figure 2. Navigating in cryo-LM for correlative studies. a) Low magnification cryo-LM view of yeast cells adhered to a sample grid. b) The same image in (a) overlaid with a high magnification fluorescence image of an area of interest and with a filled orange polygon marking the center of the sample grid. The center consists of a group of four squares, three having an extra metal tab and the fourth open. For the three squares with an extra metal tab, two pairs share the tab about the long and short axes forming an asymmetric center. This asymmetric center can be seen in the upper left corner and was used to indicate the rotation angle and handedness of the grid between cryo-LM and cryo-EM. From one low magnification image many areas of interest can be marked and used as a reference map to locate identical areas in cryo-EM. c) A magnified fluorescence cryo-LM image of the area of interest in (b). HTA1-CFP, a c-terminal CFP histone marker, can be seen as a green punctate structure labeling the location of the nucleus. d) A cryo-EM image of the corresponding area in (c). Scale bars represent 50 microns.

Figure 3. Correlation of cryo-LM and cryo-EM. Two fields of view depicting identical yeast cells correlated with cryo-bright field (a,d), cryo-fluorescence (b,e), and cryo-EM (c,f). Scale bars represent 5 microns.

Discussion

Using our cryogenic stage and sample preparation techniques, correlative studies between cryo-LM and cryo-EM exhibit several benefits over traditional room temperature approaches including, but not limited to: rapid fixation^{1,2}, reduced photobleaching³, and scanning of sample integrity prior to cryo-EM^{4,5} or chemical fixation/embedding⁶. One of the bottlenecks for cryo-EM data collection is the time required for both transferring the sample into a TEM and scanning the grid to find suitable regions to image. With cryo-LM's rapid data acquisition⁵, time spent finding good cells can be greatly reduced. Thus, we can save both time and money and mitigate this bottleneck by pre-scanning and mapping areas of interest in cryo-LM.

More expensive or complex cryogenic stages may allow higher resolution cryo-LM imaging. However, the stage fabricated here is more than adequate for many applications. If cryo-LM imaging and sample grid transfers are performed with caution as described in the text and video, fully contamination free imaging can be accomplished. This method allows direct correlation of fluorescent and electron microscopy data of the same cell, organelle or macromolecular complex dispersed on the carbon support or contained within a thin tissue section⁷. It should be noted that the value of this cryogenic stage extends beyond cryo-LM/cryo-EM correlative studies to the field of light microscopy as a whole. This cryogenic stage is well suited for both delicate samples and fluorophore dyes/stains⁸, Quantum Dots, or fluorescently tagged proteins⁹ that may be unstable during room temperature chemical fixation and embedding with glutaraldehyde and paraformaldehyde. As a result, we hope that this cryogenic stage will provide greater access to those interested in cryo-LM, who have been previously deterred by cost and insufficient access.

Troubleshooting Cryo-Light Microscopy

1. Sample Adsorption: Different samples can exhibit variable adsorption characteristics to the carbon support. You may find it necessary to use other types of support grids such as continuous carbon, holey carbon, or formvar coated grids for sample support. Additionally, if the sample is binding to the copper bars of the grid and not the carbon surface, it may be necessary to adjust the carbon surface chemistry by incubating the grid with a wetting agent or by glow discharging prior to sample addition.

2. Stage stability: If you notice vibration, it is likely due to the sample stage being overloaded with weight rather than the bubbling of IN2. The added weight of the cryogenic stage, can sometimes cause low frequency vibrations to transmit along the standard 3-axis travel microscope stage, and negatively affect imaging via blurring. This can easily be corrected by supporting the microscope's travel stage from underneath with either an adjustable mini-lift or an adjustable adapter with dual-screw arms, as shown in the video. Both of these adjustable support mechanisms interact with a non-moveable portion of the travel stage and therefore still permits X and Y-axis translation as required for imaging.

Disclosures

The authors declare no competing financial interests.

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