Common Vitrojet Standard Operating Procedure

version 1.0

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1. Purpose

1.1. Setting up, operating, and shutting down the Cryosol/Nanosoft Vitrojet in a safe and effective manner for the preparation of frozen cryo-EM samples.

2. Definitions:

2.1. Cryosol/Nanosoft Vitrojet is a vitrification device for freezing protein samples for CryoEM analysis.
2.2. Liquid Nitrogen (LN2) is a cryogenic liquid stored under pressure.
2.3. Ethane is classified as a flammable gas.

3. Supplies & Equipment

- ❑ PPE
  - ❑ Laboratory coat
  - ❑ Nitrile gloves
  - ❑ Goggles / Safety glasses
  - ❑ Face mask
- ❑ Chemicals/Reagents
  - ❑ Liquid nitrogen
  - ❑ EMAG detergent
  - ❑ 70% ethanol
  - ❑ Distilled water
  - ❑ Sample to be vitrified
- ❑ Filter paper (Whatman #1)
- ❑ Gilson positive displacement tips
- ❑ EM grids
- ❑ 60 mL syringe
- ❑ C-clips and clip rings
- ❑ Vitrojet autogrid boxes
- ❑ Autogrid storage box tool
- ❑ LN2 Dewars for Transfer
- ❑ Forceps (fine tip and long tweezers)

4. Procedure:

4.1. Gather all PPE and supplies

4.2. If necessary, turn on Vitrojet
4.2.1. If the UPS is not on, turn it on by pressing the power button on the front panel
4.2.2. If the control PC mounted to the back of the Vitrojet is not on, turn it on by pressing the power button
4.3. Clipping grids and inserting into Vitrojet
4.3.1. Remove autogrid holder from Vitrojet and rotate through all grid positions to ensure they are

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empty. Discard any old autogrids from the holder. Place holder on clean surface.

4.3.2. Clip grids using clip rings and c-clips at room temperature. **Place grid in clip ring so that the foil (carbon/gold) is facing down**.

4.3.3. Transfer autogrids from clipping station to Vitrojet autogrid holder. **Place autogrids in holder so that the foil (carbon/gold) is facing right, following the diagram on the holder**.

4.3.4. Rotate Vitrojet autogrid holder back to the home position and insert into Vitrojet. A click will be heard when it is correctly seated within the instrument.

Vitrojet grid cassette. Grids are placed so that the foil is facing to the right

4.4. Prepare bath sonicator for cleaning pin drum

4.4.1. Remove both beakers from sonicator and discard any solutions present.

4.4.2. Remove bath sonicator from frame and discard water in chamber. Wipe down with ethanol and fill chamber with fresh DI water.

4.4.3. Fill one beaker with fresh DI water and the other beaker with fresh 70% ethanol.

4.4.4. Add three drops of EMAG detergent to water and mix.

4.4.5. Replace bath sonicator in frame and place beakers back into position.

4.4.5.1. Ensure that power and nitrogen lines are properly seated.

4.5. Start Vitrojet software and initialize system

4.5.1. Ensure that ethane and compressed nitrogen tanks are open and all valves along the flowpath are open. Both gases should be delivered at ~5 bar (>70 psi).

4.5.2. On the control PC, start the Vitrojet software by double clicking the "Vitrojet GUI" icon. This will bring up both the GUI and camera interface.

4.5.3. The Vitrojet software will guide you through the preparation steps for the desired workflow. Standard use will be deposition at 4 ºC with vitrification. Press the "Start Workflow" button to begin instrument preparation.

Vitrojet software start icon

4.6. Load clipped grids into Vitrojet

4.6.1. If not done in step 4.3, clip grids and load grid cassette following the directions in step 4.3. If grids were already loaded, the cassette will need to be removed and reinserted before the "Continue" button will become available. Press the "Continue" button to proceed.

4.7. Clean pin drum

4.7.1. When instructed by the software, remove pin drum by pulling straight out.

4.7.2. While holding the pindrum upright with the pins facing upward, use a thumb and forefinger to grip the rubber pegs on each side and depress and rotate the pin drum so that the pins are lined up with the holes in the pin drum.

4.7.3. Slide pin drum into cleaning frame with pins facing downward. Position pin drum holder over beaker with DI water/detergent mix and lower the lever on the left side of the frame to drop the tips of the pins into the water.


Misaligned (left) and properly aligned (right) pins in pin drum

4.7.4. Begin sonication by flipping the power switch to the on position and turning the timer dial to 5 minutes to clean pins in water.

4.7.5. Position pin drum holder over the beaker containing 70% ethanol, turn timer to five minutes to wash pins in ethanol.

4.7.6. Turn off sonicator and carefully position pin drum over the drying area on the far right of the frame. Lower the lever on the right side of the frame to open nitrogen flow. Allow pins to dry under nitrogen for three minutes.

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4.7.7. Raise both levers to stop nitrogen flow and retract pins into drum. Place sliding pin drum holder in between beakers and pull out pin drum. Wipe pin drum surface dry with lint-free wipes and place pin drum back into Vitrojet. Two clicking sounds should be made as each side of the pin drum snaps into place. Press the "Continue" button in the Vitrojet GUI.

4.8. Empty condenser and fill humidifier

4.8.1. Following the guidance in the GUI, open the condenser valve and use an empty syringe to drain condenser. Close the condenser valve afterwards.

4.8.2. While holding a beaker or paper towels under the overflow port, fill humidifier with ~50 mL of DI water, or until water comes out of the overflow port. Follow up with ~10 mL of air to minimize leaking.

4.8.3. Use paper towels to absorb any water that may have accumulated around condenser/humidifier fill port. Press the "Continue" button in the Vitrojet GUI.

4.9. Place gridboxes in cryomodule

4.9.1. When prompted, the Vitrojet door will open and the cryomodule will slide out. Ensure that nothing is obstructing the door

4.9.2. The cryomodule is on a sensitive stage. Do not place anything on top of the cryomodule, lean on the cryomodule, or bump into the cryomodule with anything.

4.9.3. Vitrojet gridboxes are keyed and have three notches on them and the middle notch should be facing away from the Vitrojet and towards the user. Grab a gridbox with the lid on with a gridbox tool and place into the leftmost empty slot in the cryomodule.

4.9.4. Rotate lid to remove from gridbox and gently press down on the gridbox to secure it in place.

4.9.5. Repeat above steps for additional gridboxes. Press the "Continue" button in the Vitrojet GUI when all gridboxes have been placed.

4.10. Filling LN2 reservoir

4.10.1. **Avoid bumping into the cryomodule with anything in the following steps!**

4.10.2. When prompted, remove the cap on the LN2 reservoir by rotating it towards the open position and lifting.

4.10.3. The reservoir holds about 2L of LN2. Fill with approximately one liter, then wait for rapid boiling to calm down. Then slowly fill until the Vitrojet begins beeping. Replace cap on reservoir and rotate to the closed position. **It is not necessary to press down on the cap.**

4.10.4. Press the "Continue" button in the Vitrojet GUI once the cap is securely in place.

4.11. Ethane preparation, gripper homing, and LN2 refill

4.11.1. The Vitrojet will proceed to prepare the ethane cup. This process takes about 15 minutes.

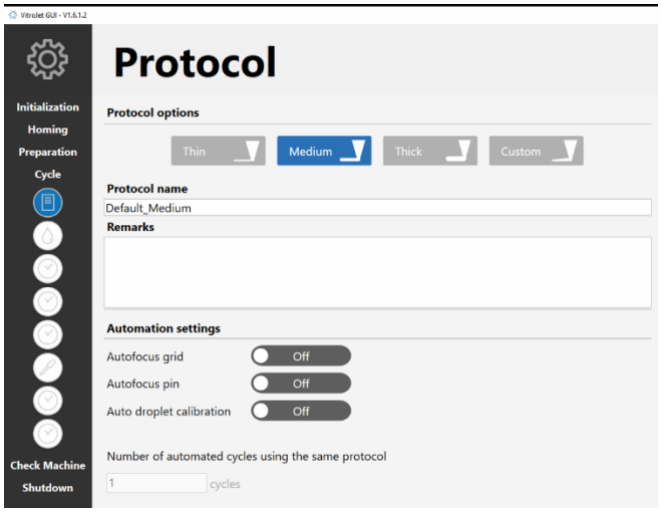
4.11.2. Once ethane is prepared, the Vitrojet will home and dry the gripper and calibrate the jet position.

4.11.3. Once temperatures have stabilized, the Vitrojet will prompt the operator to top off the LN2 reservoir. Follow the steps described above in 4.10.

4.11.4. A final ethane condensation step will occur, after which the GUI will proceed to the protocol page.

4.12. Deposition protocol configuration

4.12.1. Once all temperatures have stabilized, the protocol page will be displayed. It is at this point that humidity control in the climate chamber is initiated, so wait about five minutes for >90% humidity to be reached. This step is only necessary before vitrifying the first grid.



Basic deposition protocol settings

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4.12.2. The Vitrojet has three default deposition protocols designed to deliver thin, medium, and thick ice. One of these can be selected at the top of the protocol page.

4.12.3. Custom deposition parameters can also be entered by checking the "Show parameters" box and making any necessary adjustments.

4.12.4. To enable automated grid focusing, pin focusing, and/or sample droplet calibration, click on the appropriate toggles.

4.12.5. To prepare multiple grids with the same settings and sample automatically, the number of automated cycles can be adjusted. To have the ability to make changes to the deposition protocol for each grid, keep this value at 1.

Custom deposition protocol settings

4.12.6. If using a custom deposition protocol and not one of the three built-in ones, enter a name for the protocol. Likewise, a brief description of the protocol can be entered in the remarks section.

4.12.7. Finally, deposition protocols can be saved or loaded by using the buttons at the bottom of the protocol page.

4.12.8. Once the deposition protocol has been set, press the "Continue" button in the GUI.

4.13. Sample definition

4.13.1. The sample page will be displayed after the deposition protocol page. On this page, you can choose to load a new sample and enter a name and remarks for the sample to be deposited.

4.13.2. To load a new sample, click the "Load new sample" toggle on. This will be on by default for the first grid in a session. If this option is turned off, the currently loaded sample will be used.

4.13.3. Once all relevant sample information has been entered, press the "Continue" button in the GUI.

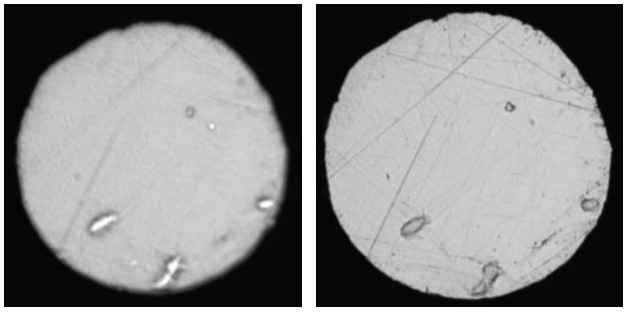
4.14. Grid plasma cleaning and grid/pin focusing

4.14.1. After progressing past the sample page, the Vitrojet will load and plasma clean the grid for the duration specified in the protocol.

4.14.2. If automatic pin or grid focusing were turned on in the protocol, the instrument will find focus for each automatically and ask for intervention if there are any problems.

4.14.2.1. If automatic focusing is not enabled in the protocol, you will be asked to focus the pin and the grid.

4.14.2.2. To correctly focus the pin, adjust focus in the GUI until the edge of the pin appears sharp. To correctly focus the grid, adjust focus in the GUI until the edges of the holes in the foil appear sharp. For either, toggle on the zoom option for a more detailed view. Press the "Continue" button to proceed.



Improperly (left) and properly (right) focused pin

4.15. Pipette tip and sample loading

4.15.1. If the option to load a new sample was enabled in the deposition protocol, the Vitrojet GUI will guide you through the steps to load a new pipette tip. Otherwise the currently loaded sample will be used. For the first grid in a session, it is important to enable this option.

4.15.2. Follow the directions presented in the GUI, ensuring that the tip is firmly placed and secured on the pipette before pressing the pipette button.

4.15.3. Continue following the guidance in the GUI to draw up the sample in to the pipette and load the

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pipette into the Vitrojet. Ensure that the outside of the tip is dried with filter paper before inserting the pipette into the Vitrojet.

4.16. Sample pickup and deposition

4.16.1. If the option to automatically calibrate the droplet was enabled in the deposition protocol, the Vitrojet will attempt to pipette out a droplet to be picked up by the pin.

4.16.1.1. If automatic droplet formation isn't enabled, adjust the pipette position in the GUI until a dome-shaped droplet forms outside of the pipette tip. Press the "Continue" button in the GUI afterwards.

4.16.2. The Vitrojet will automatically dip the pin into the droplet and deposit the sample onto the grid surface. Afterwards, it will automatically vitrify the grid and store it in a gridbox. A movie of the deposition captured on the gridcam will be played on loop in the GUI.

4.17. Vitrification cycle end

4.17.1. After placing the vitrified grid into a gridbox, the GUI will offer the option to proceed to a new cycle or to end the current session.

4.17.1.1. To proceed to a new cycle, press the "Next cycle" button and return to step 4.12

4.17.2. To end the current session, press the "End session" button. The GUI will guide you through the shutdown procedure.

4.17.3. Once the drawer has been lowered and the cryobox moved out, attach a gridbox lid to a gridbox too. Insert the lid into the LN2 in the cryomodule next to the boxes with grids to cool the lid down to LN2 temps. Then place the lid on a gridbox, screw it closed, and remove the box and put into LN2 storage. Repeat this for any other gridboxes in the cryomodule.

4.17.4. Drain the condenser following the instructions in step 4.8.1. Press the "Continue" button in the GUI.

4.17.5. Clean the pin drum following the instructions in step 4.7. Press the "Continue" button in the GUI.

4.17.6. Follow the guidance in the GUI to remove the pipette tip and reinsert pipette without a tip. Press the "Continue" button in the GUI to initiate the bakeout.

4.18. Close nitrogen and ethane valves following the instructions of the facility housing the Vitrojet. The instrument can be left to complete the bakeout on its own.

5. Chemicals:

5.1. Ethane
5.2. Liquid Nitrogen
5.3. Ethanol 70%

6. Waste Disposal:

6.1. Follow facility procedure for proper disposal **(see site specific instructions)**.
6.2. Biohazardous waste will be collected in designated bins lined with red biohazard bags.
6.3. Chemical hazardous waste will be segregated by hazard class (e.g. flammable, corrosive) and state (e.g. solid, liquid), appropriately labelled, and placed in the laboratory's hazardous waste collection.

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