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Title: A Sample Preparation Pipeline for Microcrystals at the VMXm Beamline

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

   If **Yes**, can you record movies/images using your own microscope camera? **Yes**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 19
Number of Shots: 50

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Adam Crawshaw:** Preparation of the microcrystals makes it difficult to achieve a good signal-to-noise during X-ray diffraction experiments. This protocol aims to reduce the sources of background from crystals in a controlled manner.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

1.2. **Adam Crawshaw:** The use of plunge freezing robotics and cryo-TEM grids provides a robust platform for manipulating the microcrystals repeatably, reducing the surrounding liquid volume, and providing rapid vitrification of the sample.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested B-roll: 3.4.2*

OPTIONAL

1.3. **Adam Crawshaw:** The dexterity needed to handle grids is similar to that needed for traditional crystal harvesting. Determination of initial blotting parameters using the crystallization solution is key to reducing sample usage.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested B-roll: 2.3.2*

Protocol

2. Instrument and Blotting Parameter Set-up

2.1. To begin, set up and cool the automated plunge freezer according to the manufacturer's instructions **[1].** Just before use, glow discharge the cryo-TEM *(T-E-M)* grids for 25 seconds with a 15-milliampere current and 0.39-millibar pressure **[2]**, then keep the glow discharged grids in a covered Petri dish **[3-TXT]**.

2.1.1. WIDE: Talent setting up the automated plunge freezer.

NOTE: Two takes for two different types of action were filmed for shot 2.1.1

2.1.2. Talent glow discharging the grids.

NOTE: Glow discharge is visible at 01:30

2.1.3. Talent placing the grids in a covered Petri dish. **TEXT: Repeat glow discharge, if 30 min lapses**

2.2. Set the relative humidity of the sample chamber to 90% and the blotting time to 5 seconds. Ensure that the plunge freezer is set to automatically plunge the sample after blotting is complete **[1]**.

2.2.1. Talent setting the humidity and blotting time of sample chamber.

NOTE: Shot 2.2.2 was removed.

2.3. Open the seal of the crystallization well and reservoir **[1]**. Quickly add 2 to 5 microliters of the reservoir solution in the crystal drop to maintain the volume of the drop **[2]**. Transfer 10 microliters of the reservoir solution to a 0.5-milliliter tube for later use **[3]** and reseal the well to prevent the crystallization drop from drying **[4]**.

2.3.1. Talent opening the seal of crystallization well and reservoir.

2.3.2. Talent adding reservoir solution in the crystal drop.

2.3.3. Talent transferring 10 µL reservoir solution to a 0.5 mL tube.

2.3.4. Talent sealing the well.

2.4. Use the plunge freezing forceps to pick a single glow discharged grid **[1]** and load the grid in the instrument with the carbon side facing away from the blotting arm. Rotate the forceps holding the grid so that the carbon side faces the blotting arm **[2]**.

2.4.1. Talent picking glow discharged grid with plunge freezing forceps.

2.4.2. Talent loading the grid in the instrument and rotating it. *Videographer: This shot will be used again in 3.1.3.*

2.5. Use a 2.5- microliter pipette to apply 2 microliters of reservoir solution to the non-support side of the cryo-TEM grid **[1]**. Rotate the grid with the carbon side facing away from the blotting arm **[2]** and carefully apply the reservoir liquid to the carbon-film support side of the grid **[3].** *Videographer: This step is important!*

2.5.1. Talent applying 2 µL of reservoir solution to non-support side of the grid. *Videographer: This shot will be used again in 3.1.4.*

2.5.2. Talent rotating the grid.

2.5.3. Talent applying reservoir liquid on the grid.

2.6. Initiate the blotting process **[1]** and observe the liquid drawn from the carbon surface until the wave of popping across the surface of the grid is visible **[2]**. If the wave of popping is not visible, increase the blotting time by 1 to 2 seconds **[3]** before the blotting arm retracts from the grid **[4]**. *Videographer: This step is important!*

2.6.1. Talent pressing the switch to start the blotting process.

NOTE: Action in shot 2.6.1 ends at 00:10

2.6.2. The liquid is drawn from the grid, and the wave of popping being formed. *Videographer: This shot will be used again in 3.4.1.*

NOTE: Audio slate. Tow takes for shot 2.6.2. Do not use take 1 as grid was not in focus. Use take 2. The light was low due to limited space. Higher ISO was used and file is noisy.

2.6.3. Talent increasing the blotting time.

2.6.4. Blotting arm retracting from the grid.

NOTE: For shot 2.6.4, use 2.6.1 take 2 from 00:12 to end of action.

3. Crystal Harvest Process

3.1. Place the crystallization plate under the light microscope **[1]** and position the target well within the field of view **[2]**. Place a fresh glow discharged grid in the plunge freezer and apply the reservoir liquid to the non-support side of the grid, as previously demonstrated **[3]**.

3.1.1. Talent placing the crystallization plate under the light microscope.

3.1.2. LAB MEDIA: 3.2.1: 00:04 to 00:09

3.1.3. *Use 2.4.2*

NOTE: For shot 3.1.3, use 2.4.2

3.1.4. *Use 2.5.1*

Videographer NOTE: For shot 3.1.4, reuse 2.4.3 but there is not any 2.4.3 shot.

3.2. Rotate the grid with the carbon-film support side facing the sample port of the plunge freezer **[1]**.

3.2.1. Talent rotating the grid.

3.3. Peel the temporary seal from the crystallization plate **[1]** and use the pipette set at 2 microliters to gently aspirate the crystallization drop repeatedly **[2]**. Transfer 2 microliters of the aspirated microcrystal slurry to the plunge freezer **[3]** and apply all the sample to the carbon side of the cryo-TEM grid **[4]**. *Videographer: This step is important!*

3.3.1. Talent removing the seal.

3.3.2. LAB MEDIA: 3.3.2: 00:04 to 00:017

NOTE: Video editor: Please speed up the video for shot 3.3.2

3.3.3. Talent applying 2 µL of the aspirated microcrystal slurry to the plunge freezer.

3.3.4. Talent applying sample to the carbon side of the cryo-TEM grid.

NOTE: Audio slate for shot 3.3.4

3.4. Initiate the blotting for 1 to 2 seconds and observe the wave of popping **[1]**, then immediately initiate the plunge-freezing **[2]**. Quickly transfer the grid from the liquid ethane to the grid box immersed in liquid nitrogen **[3]**. *Videographer: This step is important!*

3.4.1. *Use 2.6.2*

3.4.2. Talent initiating the plunge freezing.

3.4.3. Talent transferring the grid from liquid ethane to the grid box immersed in liquid nitrogen.

NOTE: Audio slate. Tow takes for shot 3.4.3. Take 1- some shadows due to limited space to light shot effectively. Take 2- limited space to light shot effectively, exposure increased 1 stop to reduce shadows but footage has strong highlights.

4. Light Microscopy for Sample Density Assessment

4.1. After blotting and plunge freezing the grid, retract the plunged grid from the liquid ethane by resetting the plunge-freezer **[1]**.

4.1.1. Talent retracting the plunged grid from the liquid ethane.

4.2. Remove the forceps holding the grid from the plunge freezer **[1]** and place the grid under the light microscope **[2]**. Adjust the fine focus and assess the density of the crystals across the grid **[3].**

4.2.1. Talent removing the forceps holding grid from the plunge freezer.

4.2.2. Talent placing the grid under the light microscope.

4.2.3. LAB MEDIA: 4.2.3: 00:02 to 00:16

NOTE: Video editor: Please speed up the video for shot 4.2.3.

5. Scanning Electron Microscopy for Sample Assessment

5.1. After loading the cryo-TEM grid in the SEM *(S-E-M)*, align the sample and turn on the electron beam. Initially, assess the whole grid at a 45X magnification and record the image **[1]**. Then, increase the magnification for closer inspection of individual grid squares until the individual crystals are clearly observed **[2]**.

5.1.1. LAB MEDIA: 62306_screenshot_1: 00:02 to 00:08

5.1.2. LAB MEDIA: 62306_screenshot_1: 00:15 to 00:27 and 01:36 to 01:58 *Video editor: Please speed up the video.*

NOTE: Initially, 3 shots were written for step 5.1. Shot 5.1.1 was not performed/taken during shoot and hence, removed. Now, 5.1.2 is changed to 5.1.1 and 5.1.3 is changed to 5.1.2.

5.2. Move around the grid and capture the still images **[1]**, ensuring that the grids are flat and the carbon support film is largely intact **[2]**. Ensure that there are numerous single crystals with a narrow halo of vitrified liquid surrounding the crystal, and the holes are visible in the carbon support film **[3]**. *Video editor: This step is important!*

5.2.1. LAB MEDIA: 62306_screenshot_1: 02:14 to 02:51 *Video editor: Please speed up the video.*

5.2.2. LAB MEDIA: 62306_screenshot_1: 04:05 to 04:12

5.2.3. LAB MEDIA: 62306_screenshot_1: 01:59 to 02:02.

5.3. While observing and capturing the images of the grid, make sure that the large regions of vitrified liquid are absent, hexagonal ice or surface ice is not scattered across the grid, and that the crystals are not overlapping and evenly distributed across the support film **[1]**. *Video editor: This step is important!*

5.3.1. LAB MEDIA: 62306_screenshot_1: 03:24 to 03:27 and 02:49 to 02:53

6. Grid Preparation for the Diffraction Experiments at VMXm

6.1. In a large foam dewar, cool the required number of VMXm *(V-M-X-m)* sample holders loaded in the sample cartridge **[1]**. Add liquid nitrogen above the sample position in the sample loader **[2]**.

6.1.1. Talent showing the sample holders placed in sample loader.

6.1.2. Talent pouring the liquid nitrogen until it reaches above sample position.

6.2. Swiftly transfer the grid box containing microcrystal loaded grids to the grid box recess on the sample loader **[1]** and slightly unscrew the lid to keep the lid loose and rotatable **[2]**.

6.2.1. Talent transferring the grid box to the grid box recess on the sample loader.

6.2.2. Talent loosening the lid.

NOTE: Shot 6.2.3 was removed.

6.3. Lift the grid from the grid box, rotate the grid to lay the grid flat on the sample holder **[1]**. Swiftly place the pre-cooled circlip tool over the grid in the grid opening **[2]** and press the button to install the circlip **[3]**. Add liquid nitrogen approximately 1.5 centimeters above the sample holder **[4]**.

6.3.1. Talent lifting and rotating the grid and laying the grid on the sample holder.

NOTE: VO and actions for shot 6.3.1 were changed.

6.3.2. Talent placing the circlip tool over the grid.

6.3.3. Talent pressing the button.

NOTE: Shot 6.3.2 and 6.3.3 were combined and labeled as 6.3.2.

6.3.4. Liquid nitrogen above the sample holder.

6.4. Use the VMXm sample forceps to carefully lift the loaded sample holder and place it back into the sample cartridge **[1]**. Replace the lid on the cartridge **[2]**, ensuring that the pin on the top of the cartridge engages with the hole in the lid **[3]**.

6.4.1. Talent lifting the sample holder with forceps and placing it in the sample cartridge.

6.4.2. Talent placing the lid on the cartridge.

6.4.3. Engaged pin of the cartridge with a hole in the lid.

Results

7. Results: Analysis of the Microcrystals on the Grids

7.1. The scanning electron micrographs of microcrystals prepared on cryo-TEM grids showed minimum background scatter **[1]**. The grid was free from excess liquid **[2],** and a narrow halo of liquid was observed surrounding the crystals **[3]**.

7.1.1. LAB MEDIA: Figure 1

7.1.2. LAB MEDIA: Figure 1A

7.1.3. LAB MEDIA: Figure 1B

7.2. The polyhedra crystals were observed individually **[1]** as well as in clumps **[2]**. Slightly larger insulin crystals also showed some clumping **[3]** along with isolated crystals **[4]**. Very large microcrystals were also successfully mounted on cryo-TEM grids **[5]**. The holes in the carbon support film were clearly visible, indicating strong blotting **[6]**.

7.2.1. LAB MEDIA: Figure 2A *Video Editor: Please emphasize single crystals in the image.*

7.2.2. LAB MEDIA: Figure 2A *Video Editor: Please emphasize clumped crystals in the image.*

7.2.3. LAB MEDIA: Figure 2B *Video Editor: Please emphasize clumped crystals in the image.*

7.2.4. LAB MEDIA: Figure 2B *Video Editor: Please emphasize single crystals in the image.*

7.2.5. LAB MEDIA: Figure 2D *Video Editor: Please emphasize 5 large crystals in the image.*

7.2.6. LAB MEDIA: Figure 2C and D *Video Editor: Please emphasize the black holes in the central square in the image.*

7.3. Many samples required further optimization because of variation in blotting time and concentration of the microcrystals **[1]**. The grids overloaded with crystals reduced the blotting efficiency, and multiple lattices were recorded in a single diffraction image **[2]**.

7.3.1. LAB MEDIA: Figure 3

7.3.2. LAB MEDIA: Figure 3A and B

7.4. Short blotting time for highly viscous crystallization solutions can result in an overly wet sample **[1]**. For a lower-viscosity crystallization solution, a short blotting time results in steeling of microcrystals on one side of the grids **[2]**.

7.4.1. LAB MEDIA: Figure 3C

7.4.2. LAB MEDIA: Figure 3D *Video Editor: Please emphasize the left bottom of the image.*

7.5. Optimum sample preparation makes it possible to exploit the full capabilities of VMXm to collect high-quality X-ray diffraction data at the highest possible resolution with a high signal-to-noise ratio **[1]**.

7.5.1. LAB MEDIA: Figure 5

Conclusion

8. Conclusion Interview Statements

8.1. **Adam Crawshaw:** Determining initial blotting settings with the crystallization solution is key to using minimal sample. If the sample is very limited, skip the density assessment. It is better to have a diluted sample.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested B-roll: 2.5.3 and 2.6.2*

NOTE: Three takes for shot 8.1.1. Use take 3- broad not incremented. Framing was wrong in take 1 and take 2.

8.2. **Adam Crawshaw:** The samples prepared with this method can be used for microcrystal electron diffraction or focused ion beam milling prior to microED.

8.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera