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Title: Scanning Transmission Electron Microscopy Tomography in Virology: 3D Imaging of High-Pressure Frozen, Freeze-Substituted Samples

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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**

   2.3.1, 2.4.2, 3.8.3

   Videographer: Please film the SCOPE shots using the scope kit

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

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

Current Protocol Length
Number of Steps: 22
Number of Shots: 49

Introduction

1.1. **Johannes Wieland:** Viral infection can cause significant alterations in cellular ultrastructure. To visualize these changes in three dimensions, we employ STEM tomography for imaging virus-infected cells.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 2.3.1*

What are the current experimental challenges?

1.2. **Julia LaRoche:** STEM tomography is both time-consuming and labor-intensive, making optimal sample preparation essential for biological specimens such as virus-infected cells.

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

What advantage does your protocol offer compared to other techniques?

1.3. **Clarissa Read or Johannes Wieland:** Unlike conventional TEM tomography, which is limited to sample thicknesses of approximately 200 nm, our protocol enables 3D imaging of thicker sections, allowing more comprehensive visualization of virus-infected cells and their ultrastructural alterations.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 3.3.1*

How will your findings advance research in your field?

1.4. **Martin Dass:** Many oncolytic viruses are reaching clinical trials already. Science is pushing the border of cancer treatment, and methods like STEM tomography contribute to new findings.

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

What research questions will your laboratory focus on in the future?

1.5. **Clarissa Read:** We will continue to answer virological questions by using STEM tomography and combining it with new preparation protocols and other microscopy techniques.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 4.2.1*

Protocol

Videographer's NOTE: There is no atmo-sound from 2.1.1. to 2.5.4. In case you need the atmo-sound, please take:

Script	file	Take SOUND from file
2.1.1. to 2.2.3.	From 0024 to 0033	0048
2.2.4.	0034	0073
2.4.1-2.5.4	0035-0043	0070

2. Procedure to Perform High-Pressure Freezing

Demonstrator: Johannes Wieland

2.1. To begin, fill the LN-box with liquid nitrogen in preparation for handling and transferring the frozen sapphire disks from the high-pressure freezing or HPF *(H-P-F)* holder to the storage capsules **[1]**. Keep a dewar filled with liquid nitrogen nearby to refill the LN-box as needed during the freezing process **[2]**.

2.1.1. WIDE: Talent filling liquid nitrogen into the LN-box.

2.1.2. WIDE: A shot of the dewar placed near the LN-box.

2.2. Keep a pair of insulated tweezers ready **[1]**. Prepare the sample containers that will hold the sapphire disks after HPF. Make sure that they have a lid and several holes for liquid nitrogen to pour in and small weights to ensure that they do not float **[2]**. Place the label container in the LN-box to precool before using **[3-TXT]**. Then, place the samples in the incubator **[4-TXT].**

2.2.1. Talent placing insulated tweezers next to the LN-box.

2.2.2. A shot of the prepared sample containers that will hold the sapphire disks, ensuring they have holes, lids, and small weights.

2.2.3. Talent placing the containers into the LN-box for precooling.

2.2.4. Talent bringing the samples to the high pressure freezer and putting them in the

incubator. **TXT: If not prefixed, keep samples in incubator or on heated plate**

2.3. Inspect the sapphire disks under an inverted light microscope to ensure enough cells and good cell quality before starting high-pressure freezing **[1]**.

2.3.1. SCOPE: The sapphire disks being observed under an inverted microscope.

Videographer: Please film the SCOPE shots using the scope kit

Videographer's NOTE: find on the folder Jove 68568 scopes (121009 or 115324). If possible, turn the picture 90 degrees, so that they fill the screen

2.4. To perform HPF, take one sapphire disk from the culture dish and quickly dab it on filter paper to remove excess media **[1-TXT]**. Place the disk in the HPF holder with the carbon-coated side facing upwards, ensuring the number 2 is readable **[2]**.

2.4.1. Talent using tweezers to pick a sapphire disk and quickly blotting it on filter paper. **TXT: Do not let the cells dry out**

2.4.2. SCOPE: Talent placing the disk into the HPF holder with "2" facing up.

2.5. Dip a gold spacer into hexadecane **[1]** and place it gently on the sapphire disk **[2]**. Position a second sapphire disk on top of the spacer with the carbon-coated side facing downwards to form a sandwich configuration with the cells pointing towards each other. Ensure there is no air trapped **[3-TXT]**. Close the HPF holder carefully and quickly to reduce stress on the sample **[4]**.

2.5.1. Talent dipping a gold spacer into hexadecane.

2.5.2. Talent placing the dipped gold spacer onto the disk.

2.5.3. Talent positioning a second disk on top of the spacer with the carbon-coated side facing downwards to form the sandwich with the cells pointing towards each other. **TXT: Sapphire disk – gold spacer – sapphire disk sandwich**

2.5.4. Talent closing the HPF holder.

2.6. Insert the HPF holder into the high-pressure freezer **[1]**, lock it according to the manufacturer's instructions **[2]**, and initiate the freezing process **[3]**.

2.6.1. Talent inserting the HPF holder into the high-pressure freezer. **NOTE**: 2.6.1 TO 2.6.3 COMBINED

2.6.2. Talent locking the HPF holder into the freezer.

2.6.3. Talent pressing the start button.

2.7. Next, transfer the HPF holder to the LN box **[1]**. Then, transfer the tip carrying the

sapphire disks into the precooled LN-box **[2]**. Carefully extract the disks from the holder **[3]**.

2.7.1. Talent transferring the holder from the HPF to the LN box. **Videographer's NOTE:** file 0045 (TC0.18) or 0044 (TC.0.35)

2.7.2. Talent placing the tip into the LN-box. **NOTE**: 2.7.2 and 2.7.3 COMBINED

2.7.3. Talent using tweezers to remove frozen disks from the holder.

3. Freeze-Substitution and Embedding Procedure

Demonstrator: Julia La Roche

3.1. ~~Fill 24 cryovials with the prepared freeze-substitution solution **[1-TXT]** and~~ Place the closed cryovials filled with freeze-substitution solution into a freeze-substitution device precooled to minus 90 degrees Celsius. Wait until the solution reaches the same temperature **[1-TXT]**.

~~3.1.1. Talent filling a cryovial with the freeze-substitution solution.~~ **NOTE**: Not filmed, VO merged

3.1.2. Talent inserting cryovials into the freeze-substitution device and cooling it down. **TXT: Freeze-substitution solution: 0.1% uranyl acetate, 0.2% OsO_4, and 5% water in acetone**

3.2. Precool tweezers, a scalpel, and pliers or large tweezers by immersing them in liquid nitrogen for use in transferring the sapphire disks **[2]**.

3.2.1. Talent immersing tools in liquid nitrogen for precooling.

~~3.3. Using precooled large tweezers, transfer the storage capsules containing the high-pressure frozen samples from the storage tank into the LN-box **[1]**.~~

~~3.3.1. Talent retrieving storage capsules from a storage tank and placing them into the LN-box using precooled large tweezers.~~

3.4. Transfer the high-pressure frozen samples into the freeze-substitution device. Separate the sapphire disks one by one while keeping them in liquid nitrogen **[1]** and quickly transfer each disk individually into a cryovial with freeze-substitution solution, ensuring that the disk is fully immersed **[2].** After closing the cryovial, immediately place it back into the freeze-substitution device **[3].** Once all samples are placed, start the substitution process **[4].**

3.4.1. Talent separates the sapphire disks.

3.4.2. Talent transfers the disk into a cryovial with freeze-substitution solution. **NOTE**:

3.4.2 and 3.4.3 COMBINED

3.4.3. Talent placing the cryovial into the freeze-substitution device.

3.4.4. Shot of all the samples placed in freeze-substitution device.

3.5. As soon as the samples reach 20 degrees Celsius, continue with washing the samples 3 times for 30 minute each with 100 % acetone **[1].**

3.5.1. Close-up: Sample washing process using a plastic pipette.

3.6. To pre-embed the samples**,** incubate the samples in 1 by 3 epoxy resin and 2 by 3 acetone for 1 hour **[1].** After incubating the samples with 2/3 epoxy resin and 1/3 acetone for 3 hours, exchange the solution with 100 % epoxy resin and incubate overnight with the vial lid open to allow remaining acetone to evaporate **[2].**

3.6.1. Talent adding epoxy resin to the sample using a plastic pipette.

3.6.2. Talent removing epoxy acetone solution and adding 100% epoxy and keeping it aside with open lid.

3.7. On the following day, transfer the sapphire disks into new, labeled reaction tubes filled with freshly prepared epoxy resin. Position the disks horizontally, supported by the wall of the tube, ensuring that the side with cells is facing upward **[1]**. Then, place the reaction tubes with the lids open in an oven set to 60 degrees Celsius and allow the resin to polymerize for at least 48 hours **[2]**.

3.7.1. Talent placing sapphire disks into labeled reaction tubes filled with epoxy resin, ensuring the disks are positioned horizontally, supported by the wall of the tube, and the side with cells is facing upward.

3.7.2. Talent placing the open reaction tubes into an oven.

3.8. After the epoxy resin has polymerized, immerse the tip of a closed reaction tube in liquid nitrogen for approximately 10 seconds **[1]**. Hit the reaction tube on a table to release the resin block **[2]**. The sapphire disk will separate easily from the carbon coat, which remains attached to the embedded cells **[3]**.

3.8.1. Talent immersing the reaction tube tip into liquid nitrogen.

3.8.2. Talent tapping the reaction tube sharply on the table.

3.8.3. SCOPE: Resin block separating from sapphire disk, with carbon coat (attached to the embedded cells) visible. **Videographer NOTE:** find in the folder Jove 68568 scopes (if possible, turn the picture 90 degrees

3.9. Select the region of interest in the embedded sample and narrow down the sample size

to the region of interest **[1].** Using an ultramicrotome with a diamond knife, collect sections of around 1 micrometer thickness on a TEM grid with a formvar film for STEM imaging and STEM tomography **[2].**

3.9.1 Talent will show the ultramicrotome and a Block before and after trimming

3.9.2. Talent will show the cutting process and the final Grid with collected sections.

4. Scanning Transmission Electron Microscopy (STEM) Tilt Series Acquisition

Demonstrator: Johannes Wieland

4.1. After cutting and inspecting ultrathin sections, the samples are ready for imaging. Based on the research question, insert either the brightfield detector, the darkfield detector, or both **[1]**. Activate the **scan** mode and acquire a test image of the sample **[2]**.

4.1.1. Talent inserting the selected detector into the microscope based on experimental needs.

4.1.2. SCREEN: JoVE68568_Wieland_4_1_2.mp4 .

4.2. Select a magnification higher than 800,000 *(eight-hundred-thousand)* times, which is larger than the final desired magnification **[1]**. Near the region of interest, adjust the focus and correct astigmatism while avoiding direct beam exposure on the critical sample areas **[2]**.

4.2.1. SCREEN: JoVE68568_Wieland_4_2_1_and_4_2_2---Trimmed.mp4 00:00-00:10.

4.2.2. SCREEN: JoVE68568_Wieland_4_2_1_and_4_2_2---Trimmed.mp4 00:11-00:19.

4.3. Set the eucentric height so the region of interest or ROI *(R-O-I)* stays centered throughout the tilt range **[1]**. Choose a feature near the ROI, bring it to the center, focus, correct astigmatism, and tilt the stage stepwise to minus 72 degrees **[2-TXT]**.

4.3.1. SCREEN: JoVE68568_Wieland_4_3_Trimmed.mp4. 00:00-00:10

4.3.2. SCREEN: JoVE68568_Wieland_4_3_Trimmed.mp4 00:11-00:00:27.

TXT: Adjust the z-position of the holder to keep the feature centered

4.4. To choose the field of view, navigate back to the ROI, bring it into focus, and capture an overview image **[1]**. Set the final magnification desired for tilt series acquisition **[2]**.

4.4.1. SCREEN: JoVE68568_Wieland_4_4_1_and_4_4_2.mp4 00:10-00:22.

4.4.2. SCREEN: JoVE68568_Wieland_4_4_1_and_4_4_2.mp4 00:00-00:09.

4.5. Set up automatic tilt series acquisition of images ranging from minus 72 degrees to plus 72 degrees and image every 1.5 degrees **[1-TXT]**.

4.5.1. SCREEN: JoVE68568_Wieland_4_5_1_1_Untrimmed.mp4 01:05-01:10 AND 01:35-01:40. **TXT: To offset mechanical backlash, begin the tilt series at a larger angle than the initial imaging angle**

4.6. Enable dynamic focus in the acquisition software and start the automated tilt series acquisition **[1]**.

4.6.1. SCREEN: JoVE68568_Wieland_4_5_1_3_and_4_6_1_Untrimmed.mp4 00:30-00:50

Results

5. Results

5.1. Representative virtual slices from the STEM tomogram illustrated how virion orientation impacts the visibility of the characteristic bullet shape in two-dimensional views **[1]**. Only virions aligned parallel to the viewing plane appeared with the full, well-defined bullet morphology **[2]**, while others appeared circular or elongated depending on their tilt angle **[3]**.

5.1.1. LAB MEDIA: Figure 5A-5C.

5.1.2. LAB MEDIA: Figure 5A-5C. *Video Editor: Highlight C.*

5.1.3. LAB MEDIA: Figure 5A-5C. *Video Editor: Highlight A and B.*

5.2. To overcome the orientation limitations of 2D imaging, STEM tomography was used to capture large clusters of budding recombinant vesicular stomatitis virus virions, enabling assessment of virion shape in three dimensions without directional bias **[1]**.

5.2.1. LAB MEDIA: Figure 5D.

5.3. The side view of the tomogram confirmed the volumetric advantage of STEM tomography, capturing many complete virions within a single 600 nanometer-thick section **[1]**, in contrast to the limited sampling possible with a 200 nanometer TEM section **[2]**.

5.3.1. LAB MEDIA: Figure 5E.

5.3.2. LAB MEDIA: Figure 5E. *Video Editor: Emphasize the section inside the two white dashed horizontal lines.*

- dewar

Pronunciation link: https://www.merriam-webster.com/dictionary/dewar
IPA: /ˈduːər/
Phonetic Spelling: DOO-er

- sapphire (as in “sapphire disks”)

Pronunciation link: https://www.merriam-webster.com/dictionary/sapphire
IPA: /ˈsæfaɪər/ or /ˈsæfaɪr/
Phonetic Spelling: SAF-ire

- hexadecane

Pronunciation link: https://www.merriam-webster.com/dictionary/hexadecane
IPA: /ˌhɛksəˈdɛkeɪn/
Phonetic Spelling: hek-suh-DEK-ayne

- vitrification

Pronunciation link: https://www.merriam-webster.com/dictionary/vitrification
IPA: /ˌvɪtrɪfəˈkeɪʃən/
Phonetic Spelling: vit-ri-fuh-KAY-shun

- ultramicrotome

Pronunciation link: No confirmed link found
IPA: /ˌʌltrəˈmaɪkrətoʊm/
Phonetic Spelling: UL-tra-MY-kroh-tohm

- formvar (in "formvar film")

Pronunciation link: No confirmed link found
IPA: /ˈfɔːrmˌvɑːr/ or /ˈfɔːrmˌvɑr/
Phonetic Spelling: FORM-var

- tomography (as in "STEM tomography")

Pronunciation link: https://www.merriam-webster.com/dictionary/tomography
IPA: /təˈmɒgrəfi/ or /təˈmɑːgrəfi/
Phonetic Spelling: tuh-MOG-ruh-fee