

Submission ID #: 70192

Scriptwriter Name: Pallavi Sharma

Project Page Link: <https://review.jove.com/account/file-uploader?src=21408608>

Title: Cryo-Electron Tomography Workflow for Thick Tissues Demonstrated in Mouse Hippocampus

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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**

NO

Our microscope (Leica EM ICE and Leica S9i), the stereomicroscope has an HDMI interface. Using the scope kit attached to an eyepiece and performing the procedure with one eye is acceptable.

2.2.2 and 3.2.1

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**

3. Filming location: Will the filming need to take place in multiple locations? **YES**

There are three filming locations, all very close to each other—just in the next room.

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **NO**

Current Protocol Length

Number of Steps: 29

Number of Shots: 49

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Jing Wu:** My research focuses on establishing a standardized cryo-ET workflow to enable in situ structural analysis of thick tissues like the mouse hippocampus.
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 2.4.1*

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Jing Wu:** Existing methods lack reliability for thick tissues, so this protocol integrates optimized steps for standardized analysis.
 - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Jing Wu:** This protocol enables researchers to study native cellular ultrastructure directly within complex tissue environments.
 - 1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Jing Wu:** The most critical challenge in this cryo-ET protocol is achieving uniform, artifact-free vitrification of thick tissue samples.

- 1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 3.2.2*

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Jing Wu:** Following this procedure, researchers can perform subtomogram averaging and structural analysis to resolve macromolecular complexes.

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

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at SUSTech

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

NOTE: Regarding the footage, some additional shots were captured under the stereomicroscope during operation; please incorporate them during editing. Also, due to the lengthy duration of the screen recordings, all have been uploaded after being sped up.

2. High-Pressure Freezing of Thick Tissue for Cryo-electron Tomography

Demonstrator: Jinrong Hu

- 2.1. To begin, place a 10-microliter droplet of 10 percent sucrose solution on a clean glass surface [1].
 - 2.1.1. WIDE: Talent placing a 10-microliter droplet of 10 percent sucrose solution onto a clean glass surface.
- 2.2. Rapidly dissect the hippocampus from a freshly euthanized mouse [1]. Using a sharp razor blade, excise small tissue pieces [2-TXT].
 - 2.2.1. Talent rapidly dissecting the hippocampus.
 - 2.2.2. SCOPE: Talent excising small hippocampal tissue pieces with a sharp razor blade.
TXT: Tissue Size: 0.5-1 mm³ *Videographer: Please film the SCOPE shots using the scope kit*
- 2.3. Using a toothpick or fine brush, transfer the tissue onto the prepared copper support TEM grids within the high-pressure freezing carrier [1]. Using 2-methylbutane isopentane, gently maneuver the tissue into place while minimizing mechanical stress on the grid [2].
 - 2.3.1. Talent transfers the tissue onto the copper support TEM grids within the high-pressure freezing carrier using a toothpick or fine brush.
 - 2.3.2. Talent gently maneuvering the tissue into place with 2-methylbutane isopentane while minimizing mechanical stress on the grid.
- 2.4. Then, cover the tissue and grid with a 3-millimeter diameter copper foil disc [1]. To complete the assembly, place the second high-pressure freezing carrier, flat side down, on top to form a sealed waffle sandwich [2].
 - 2.4.1. Talent covering the tissue and grid with a 3 millimeter diameter copper foil disc.
 - 2.4.2. Talent placing the second high-pressure freezing carrier flat face down on top to form the sealed waffle sandwich.
- 2.5. Immediately initiate high-pressure freezing according to the manufacturer's protocol

[1], setting the pressure to approximately 2100 bar [2-TXT].

2.5.1. Talent placing the assembled sample into the high-pressure freezing device.

2.5.2. Talent setting the pressure. **TXT: Dissection-to-freezing time ≤ 90 s**

3. Focused Ion Beam (FIB) Milling and Lift-out of the Tissue

3.1. To prepare the receiver grid, obtain a new 300 mesh gold grid to serve as the final receiver grid for the lift-out procedure [1].

3.1.1. Talent holding a new 300 mesh gold grid.

3.2. Under a stereomicroscope at room temperature, gently clip the grid into a compatible cryogenic focused ion beam AutoGrid ring [1]. Then, store the assembled AutoGrid directly in a dry oven maintained at approximately 40 degrees Celsius to minimize frost accumulation before use [2].

3.2.1. SCOPE: Talent clipping the gold grid into a cryogenic focused ion beam AutoGrid ring.

3.2.2. Talent placing the assembled AutoGrid into a dry oven.

3.3. While continuously immersing the assembly in liquid nitrogen, carefully separate the vitrified TEM grid from the surrounding waffle assembly components [1]. Using pre-cooled fine-tipped tweezers, gently pry the two high-pressure freezing carriers apart [2].

3.3.1. Talent separating the vitrified TEM grid from the waffle assembly while submerged in liquid nitrogen.

3.3.2. Talent gently prying apart the two high-pressure freezing carriers with pre-cooled fine tipped tweezers.

3.4. Next, on a pre-cooled cryogenic loading workstation, mount the isolated tissue grid into a second cryogenic focused ion beam AutoGrid ring under the protection of liquid nitrogen [1].

3.4.1. Talent mounting the isolated tissue grid into a cryogenic focused ion beam AutoGrid ring on a pre-cooled cryogenic loading workstation under liquid nitrogen.

3.5. Load both AutoGrids into the designated slots of the cryogenic transfer shuttle sample holder [1]. Orient each AutoGrid so that the milling notch faces outward and is positioned vertically to enable a lower milling angle [2].

3.5.1. Talent loading the two AutoGrids into the designated slots of the cryogenic transfer shuttle sample holder.

3.5.2. Talent adjusting the AutoGrids in the shuttle so that the milling notches face outward and are vertically aligned.

3.6. Keep the fully loaded shuttle immersed in liquid nitrogen or maintained in a pre-cooled state until transfer into the focused ion beam SEM [1]. Load the sample stage into the instrument using the sample rod [2]. **NOTE: The VO is edited for the additional shot**

3.6.1. Talent holding the fully loaded cryogenic transfer shuttle submerged in liquid nitrogen.

Added shot: Talent loading the sample stage in the instrument

3.7. After cryogenic transfer into the microscope, navigate to the tissue grid at low SEM magnification [1]. ~~Identify tissue regions by their distinct contrast against the support film [2].~~ **NOTE: The VO is edited for the deleted shot**

3.7.1. SCREEN: 70192_screenshot_1.mp4: 00:00-end

~~3.7.2. **SCREEN:** Display the tissue regions appearing with distinct contrast against the support film on the scanning electron microscopy monitor. **NOTE: Not filmed**~~

3.8. Now, sputter coat the grid surface with platinum at 30 milliamperes for 15 seconds to provide conductive protection [1]. Deposit a 0.5 to 1.0 micrometer thick organometallic platinum cap using the gas injection system for approximately 60 seconds [2].

3.8.1. SCREEN: 70192_screenshot_2.mp4: 00:01-00:06, 00:16-00:20

3.8.2. SCREEN: 70192_screenshot_2.mp4: 00:25-00:45

3.9. Apply a final sputter-coated platinum layer to the grid surface at 30 milliamperes for 15 seconds [1].

3.9.1. SCREEN: 70192_screenshot_2.mp4: 00:54-01:04.

3.10. Now, using the SEM electron beam image, navigate to the selected region of interest on the tissue grid [1].

3.10.1. SCREEN: 70192_screenshot_3.mp4: 00:00-00:04

3.11. To orient the sample for front-side milling, adjust the stage to approximately 110 degrees rotation and 7 degrees tilt to present the sample surface at an angle suitable for ion beam access [1].

3.11.1. SCREEN: 70192_screenshot_3.mp4: 00:05-00:30

3.12. For trench milling, define a rectangular trench surrounding the target tissue block using the ion beam operated at 30 kilovolts [1]. Begin milling at a distance from the block using a relatively high current of 15 nanoampere to achieve rapid material removal [2].

3.12.1. SCREEN: 70192_screenshot_4.mp4: 00:05-00:28

3.12.2. SCREEN: 70192_screenshot_4.mp4: 00:30-00:35, 01:23-01:25

3.13. As the trench walls approach the block to within a few micrometers, reduce the ion beam current to 7 nanoampere to achieve more precise milling control [1]. Leave one side of the block attached to the bulk material to create a temporary holding tab [2].

- 3.13.1. SCREEN: 70192_screenshot_5.mp4: 00:00-00:15
- 3.13.2. SCREEN: 70192_screenshot_5.mp4: 02:27-02:38
- 3.14. For tissue blocks with an estimated thickness greater than 20 micrometers, perform an undercut milling step from the reverse side to facilitate the final lift out [1].
- 3.14.1. SCREEN: 70192_screenshot_6.mp4: 00:00-00:30
- 3.15. Rotate the stage and adjust the tilt to access the bottom of the block [1]. Mill the underside connection using the focused ion beam at 30 kilovolts and a current of 3 nanoampere [2]. Mill the bottom of the metal block to fit tightly against the sample block [3]. **NOTE: The VO is edited for the additional shot**
- 3.15.1. SCREEN: 70192_screenshot_6.mp4: 01:15-01:20
- 3.15.2. SCREEN: 70192_screenshot_6.mp4: 01:20-01:45
- Added shot: SCREEN: 70192_screenshot_7.mp4: 00:09-00:31**
- 3.16. To attach the sample to the needle, ensure that the gold block is in direct contact with the target sample [1]. Using the focused ion beam at 30 kilovolts and 0.5 nanoampere, perform 8 to 12 sequential cross-section milling patterns at the interface to weld the gold block to the sample [2].
- 3.16.1. SCREEN: 70192_screenshot_8.mp4: 00:07-end
- 3.16.2. SCREEN: 70192_screenshot_9.mp4: 00:15-00:45
- 3.17. To lift out the tissue block, sever the remaining tab connecting the block to the bulk sample using the focused ion beam [1].
- 3.17.1. SCREEN: 70192_screenshot_10.mp4: 00:00-00:25
- 3.18. Carefully maneuver the micromanipulator needle to lift the isolated tissue block completely clear of the trench and the surrounding grid. Retract the needle and transport the block to the receiver grid [2].
- 3.18.1. SCREEN: 70192_screenshot_10.mp4: 00:25-00:35.
- 3.19. Now, navigate the micromanipulator needle to a pre-cooled 300 mesh gold receiver grid [1].
- 3.19.1. SCREEN: 70192_screenshot_11.mp4: 00:00-00:19
- 3.20. Lower the block onto a chosen grid square [1] and bring the tissue block into contact with the film on the grid [2]. Lift the block vertically to break the contact [3]. Mill a 3 to 4 micrometer thick slice from the block onto the grid film using the ion beam [3].
- 3.20.1. SCREEN: 70192_screenshot_11.mp4: 00:19-00:25
- 3.20.2. SCREEN: 70192_screenshot_11.mp4: 00:26-00:40
- 3.20.3. SCREEN: 70192_screenshot_11.mp4: 01:11-01:17

3.20.4. SCREEN: 70192_screenshot_11.mp4: 01:53-02:00.

3.21. Now, weld the slice securely by milling using cleaning cross-section patterns on both sides of the junction **[1-TXT]**.

3.21.1. SCREEN: 70192_screenshot_12.mp4: 00:00-00:35. **TXT: Reapply the conductive and protective coating to the grid surface as shown previously**

3.22. For lamella thinning, after setting the stage tilt, define the final lamella position within a smooth area of the tissue block **[1]**.

3.22.1. SCREEN: 70192_screenshot_12.mp4: 00:14-00:23

3.23. Perform systematic milling using ion beam currents and over-tilt compensations specified in the presented Table **[1]**. When within 1 to 2 micrometers of the lamella, optionally mill a stress relief trench approximately 1.5 micrometers away to prevent bending or curling of the lamella **[2]**.

3.23.1. LAB MEDIA: Table 1

3.23.2. SCREEN: 70192_screenshot_13.mp4: 06:27-06:32, 08:07-08:15

3.24. After lamella preparation, acquire images using Cryo-electron microscopy **[1]**.

3.24.1. Talent at the Cryo-EM observing the acquired images.

Results

4. Results

4.1. Tissue blocks processed by high-pressure freezing with the “waffle” method exhibited a uniform and electron-transparent appearance [1].

4.1.1. LAB MEDIA: Figure 4C. *Video editor: Show the micrograph and highlight the four dashed yellow boxes labelled 1–4 within the image.*

4.2. Reconstructed tomograms resolved mitochondrial structures within the cellular environment [1]. Macromolecular complexes such as ribosomes were clearly resolved in the cytoplasm [2].

4.2.1. LAB MEDIA: Figure 5A and B. *Video editor: Highlight the region labeled “Mitochondria” and the yellow arrow pointing to the curved internal membrane structures.*

4.2.2. LAB MEDIA: Figure 5C and D. *Video editor: Highlight the yellow arrows pointing to the small dot-like particles labeled “Ribosome”.*

4.3. Cytoskeletal and membrane-associated cellular features [1] and nuclear pore complexes were also visible in the reconstructed tomograms [2].

4.3.1. LAB MEDIA: Figure 5E and F. *Video editor: Highlight the arrows pointing to the long filament structures labeled “Microtubule”.*

4.3.2. LAB MEDIA: Figure 5G and H. *Video editor: Highlight the arrow labeled “NPC” at the curved membrane.*

4.4. Synaptic structures and features, including calcium stores and myelin sheath structures, were clearly seen in the tomograms [1].

4.4.1. LAB MEDIA: Figure 5I and J, K and L. *Video editor: Highlight the arrow labeled “Synaptic cleft”, “Calcium stores” and Myelin sheath .*

1. Microliter

Pronunciation link: <https://www.merriam-webster.com/dictionary/microliter>

IPA: /ˈmaɪkroʊ.liːtər/

Phonetic Spelling: my·kroh·lee·ter

2. Sucrose

Pronunciation link: <https://www.merriam-webster.com/dictionary/sucrose>

- IPA: /'su:kroʊs/
Phonetic Spelling: soo·krohs
3. Hippocampus
Pronunciation link: <https://www.merriam-webster.com/dictionary/hippocampus>
IPA: /,hɪpə'kæmpəs/
Phonetic Spelling: hip·uh·kam·puhs
4. Euthanized
Pronunciation link: <https://www.merriam-webster.com/dictionary/euthanize>
IPA: /'ju:θə'naɪz/
Phonetic Spelling: yoo·thuh·nyze
5. Isopentane
Pronunciation link: <https://www.merriam-webster.com/dictionary/isopentane>
IPA: /,aɪsoʊ'penteɪn/
Phonetic Spelling: eye·soh·pen·tayn
6. Cryogenic
Pronunciation link: <https://www.merriam-webster.com/dictionary/cryogenic>
IPA: /,kraɪoʊ'dʒenɪk/
Phonetic Spelling: kry·oh·jen·ik
7. Vitrified
Pronunciation link: <https://www.merriam-webster.com/dictionary/vitrify>
IPA: /'vɪtrə'faɪd/
Phonetic Spelling: vi·truh·fyde
8. Stereomicroscope
Pronunciation link: <https://www.merriam-webster.com/dictionary/stereomicroscope>
IPA: /,sterɪoʊ'maɪkrə'skoʊp/
Phonetic Spelling: ster·ee·oh·my·kruh·skohp
9. Sputter
Pronunciation link: <https://www.merriam-webster.com/dictionary/sputter>
IPA: /'spʌtər/
Phonetic Spelling: sput·er
10. Organometallic
Pronunciation link: <https://www.merriam-webster.com/dictionary/organometallic>
IPA: /,ɔ:rgənəʊmə'tælɪk/
Phonetic Spelling: or·guh·noh·muh·tal·ik
11. Micrometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>
IPA: /maɪ'kra:mətər/
Phonetic Spelling: my·krah·muh·ter
12. Nanoampere
Pronunciation link: <https://www.merriam-webster.com/dictionary/nanoampere>
IPA: /'nænəʊ,æmpɪr/
Phonetic Spelling: nan·oh·am·peer
13. Kilovolt
Pronunciation link: <https://www.merriam-webster.com/dictionary/kilovolt>

- IPA: /'kɪləˌvoʊlt/
Phonetic Spelling: kil·uh·vohlt
14. Lamella
Pronunciation link: <https://www.merriam-webster.com/dictionary/lamella>
IPA: /lə'mɛlə/
Phonetic Spelling: luh·mel·uh
15. Tomograms
Pronunciation link: <https://www.merriam-webster.com/dictionary/tomogram>
IPA: /'toʊməˌgræm/
Phonetic Spelling: toh·muh·gram
16. Mitochondrial
Pronunciation link: <https://www.merriam-webster.com/dictionary/mitochondrial>
IPA: /ˌmaɪtoʊ'kɑːndriəl/
Phonetic Spelling: my·toh·kon·dree·uhl
17. Macromolecular
Pronunciation link: <https://www.merriam-webster.com/dictionary/macromolecular>
IPA: /ˌmækrəˌmɑː'lekjələr/
Phonetic Spelling: mak·roh·muh·lek·yuh·ler
18. Ribosomes
Pronunciation link: <https://www.merriam-webster.com/dictionary/ribosome>
IPA: /'raɪbəˌsoʊm/
Phonetic Spelling: rye·buh·sohm
19. Cytoplasm
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytoplasm>
IPA: /'sartəˌplæzəm/
Phonetic Spelling: sy·tuh·plaz·uhm
20. Cytoskeletal
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytoskeleton>
IPA: /ˌsartəʊ'skɛlɪtəl/
Phonetic Spelling: sy·toh·skel·uh·tl
21. Microtubule
Pronunciation link: <https://www.merriam-webster.com/dictionary/microtubule>
IPA: /ˌmaɪkroʊ'tuːbjʊːl/
Phonetic Spelling: my·kroh·too·byool
22. Synaptic
Pronunciation link: <https://www.merriam-webster.com/dictionary/synaptic>
IPA: /sɪ'næptɪk/
Phonetic Spelling: si·nap·tik
23. Myelin
Pronunciation link: <https://www.merriam-webster.com/dictionary/myelin>
IPA: /'maɪəlɪn/
Phonetic Spelling: my·uh·lin
24. Calcium
Pronunciation link: <https://www.merriam-webster.com/dictionary/calcium>

IPA: /'kælsiəm/

Phonetic Spelling: kal·see·uhm

25. Electron microscopy

Pronunciation link: <https://www.merriam-webster.com/dictionary/electron%20microscopy>

IPA: /ɪ'lektɹɑːn maɪ'kraːskəpi/

Phonetic Spelling: ih·lek·tron my·krah·skuh·pee