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Project Page Link: https://review.jove.com/account/file-uploader?src=20812638

Title: Workflow Using a Cryogenic Coincident Fluorescence, Electron, and Ion Beam Microscope for Targeted Milling of Cells

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

Current Protocol Length
Number of Steps: 23
Number of Shots: 41

Introduction

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

~~**REQUIRED:** What is the scope of your research? What questions are you trying to answer?~~

1.1. **Jue Wang:** My research develops optical microscopy methods to preserve rare cellular structures in cryogenic lamella during focused ion beam milling, enabling their detailed analysis by cryogenic electron tomography to uncover native biological organization.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:3.1*

What are the most recent developments in your field of research?

1.2. **Jue Wang:** Optical microscopy has recently been integrated into cryoFIB-SEM systems, but rarely at the same focal position as the FIB. This necessitates registration-based guidance approaches to preserve fluorescently labelled structures in final lamella.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:2.19*

What are the current experimental challenges?

1.3. **Jue Wang:** Accuracy of Fluorescence-based lamella targeting is limited by registration approaches, which suffer from registration error, diffraction-limited resolution, refractive index mismatch, and milling-induced motion.

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

What significant findings have you established in your field?

1.4. **Peter Dahlberg:** Simultaneous optical microscopy and FIB milling removes many of these sources of guidance error by enabling real-time feedback for precise milling that preserves fluorescently labeled structures for cryo-electron tomography within the final lamella.

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

What new scientific questions have your results paved the way for?

1.5. **Jue Wang:** Our research makes it possible to see cellular structures that were previously challenging to directly visualize by cryo-ET, shedding light on molecular mechanisms behind a wide spectrum of key macromolecular machines.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:3.4*

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

Protocol

2. High-Precision Targeting for Cryo-Correlative Light and Electron Microscopy

Demonstrator: Jue Wang

2.1. To begin, manually cool the transfer station using liquid nitrogen once the system temperature nears cryogenic levels **[1]**.

2.1.1. WIDE: Talent pouring liquid nitrogen into the transfer station while checking the temperature gauge.
Videographer's Note: I included several angles of the liquid nitrogen being poured into the transfer station, as well as the temperature gauge, which is across the room from the transfer station. I've slated them with the additional info

2.2. Load one clipped Autogrid into liquid nitrogen to cool it and then into the sample cassette designed for the cryogenic light microscopy system **[1]**. Ensure that the Autogrid is positioned between the glued coverslip and the spacer **[2]**.
NOTE: The VO has been edited to accommodate the added action in 2.2.1

2.2.1. Talent placing a clipped Autogrid into the cassette.
Videographer's Note: For 2.2.1, before placing the clipped autogrid into the cassette, the talent places the tools in the liquid nitrogen. This is to cool down the tools.

2.2.2. Shot of the Autogrid correctly seated between the glued coverslip and spacer.

2.3. Tighten the ring screw to close the cassette **[1]**, and insert it into the slot on the transfer station **[2]**.

2.3.1. Talent tightening the ring screw on the cassette.

2.3.2. ~~SCREEN~~: Talent inserting the closed cassette into the transfer station slot.
NOTE: Shot by videographer

2.4. Attach the transfer station to the cryogenic light microscopy system **[1]**. Use the internal chamber camera to guide the sample cassette onto the sample stage **[2]**.

2.4.1. Talent connecting the transfer station to the system interface.

2.4.2. ~~SCREEN:~~ Display of the internal chamber camera feed guiding the sample cassette accurately onto the sample stage.
NOTE: Shot by videographer

2.5. In the light microscopy software, move the sample stage from the Loading position to the predefined three-beam position **[1]**. In the FIB-SEM *(Fib--Sem)* software, set the to **100X** *(Hundred-Ex)* and click on the top-left window **[2]**.

2.5.1. SCREEN: 2.5.1-(1).mp4 00:08-00:24, 01:34-01:40

2.5.2. SCREEN: 2.5.2-(1).mp4 00:00-00:09

2.6. In the **Alignment** tab of the light microscopy software, click on the **z** and select the **play** function to acquire a SEM atlas of the grid **[1]**. Identify a square area with broken carbon film and double-click on it to move the sample stage to that location **[2]**.

2.6.1. SCREEN: 2.6.1-(1).mp4 00:00-00:10

2.6.2. SCREEN: 2.6.2.mp4 00:00-00:22

2.7. Return to the software interface, activate the FIB and acquire an image **[1]**. Adjust the magnification using the drop down manu on the top left corner **[2].** Press **Play** to display the FIB image **[3-TXT].** Then set the step size using the slide bar **[4].**

2.7.1. SCREEN: 2.7.1_fib_added.mp4.mp4 00:30-00:53

2.7.2. SCREEN: 2.7.1_fib_added.mp4.mp4 00:55-01:07

2.7.3. SCREEN: 2.7.2_updated-(1).mp4 00:00-00:03
TXT: Set step size using slide bar

2.8. Now click **+z** *(plus-Z)* and **-z** *(minus-Z)* to adjust the stage z position until the horizontal line present in the ion and the electrode images coincide with the same feature **[1].** Select the SEM tab in the light microscopy software **[2]**. Use the plus or minus **X** and **Y** buttons to align a common feature between the SEM and ion images **[3].**

2.8.1. SCREN: 2.7.2_updated-(1).mp4 00:12-00:42, 01:19

2.8.2. SCREEN: 2.7.3-(1).mp4 00:00-00:05

2.8.3. SCREEN: 2.7.3-(1).mp4 00:28-00:34, 01:57-02:05

2.9. Now turn on the fluorescence microscope **[1].** Select **FLM** *(F-L-M)* tab, set reflected light imaging conditions and select **play [2].** Then align the reflected light image with the SEM *(sem)* image. **[3]**.

2.9.1. Talent pressing a button on a computer screen to switch on the microscope.

2.9.2. SCREEN: 2.8.2-(1).mp4 00:00-00:12

2.9.3. SCREEN: 2.8.2-(1).mp4 00:13-00:21, 00:46-00:53

2.10. Zoom into the image and click the **rectangle** option to draw focus of the ion beam **[1].** Press **Start Patterning in Display 2** *(two)* to mill a small pattern using the focused ion beam **[1]**.

2.10.1. SCREEN: 2.9.1-(1).mp4 00:00-00:13

2.10.2. SCREEN: 2.9.1-(1).mp4 00:19-00:42, 00:58-00:59

2.11. Confirm that the milled pattern is visible in both the focused ion beam and fluorescent microscope views **[1-TXT]**.

2.11.1. SCREEN: 2.10.1-(1).mp4 00:35-00:50
TXT: If necessary, repeat alignment

2.12. In the light microscopy software, move to the **Localization** tab and activate the appropriate conditions to set up the fluorescent channels **[1]**. Assign each fluorophore to a separate channel and include an additional channel for brightfield **[2]**.

2.12.1. SCREEN: 2.11.1-(1).mp4 00:00-00:19

2.12.2. SCREEN: 2.12.1-(1).mp4 00:00-00:12

2.13. Adjust the LED *(L-E-D)* power and exposure time for each fluorescent channel to identify the target of interest **[1-TXT]**. Once the parameters are satisfactory, acquire a pre-milling fluorescent z-stack by stepping through the axial direction **[2]**. Then, mark the target of interest as a region of interest **[3]**.

2.13.1. SCREEN: 2.12.1.mp4 00:14-00:30, 00:55-01:04
TXT: Use 1.2 s and 30 mW when imaging SiR-tubulin

2.13.2. SCREEN: 2.12.2-2.12.3-(1).mp4 00:00-00:10, 00:25-00:30, 01:0-01:30

2.13.3. SCREEN: 2.12.2-2.12.3-(1).mp4 01:40-01:45

2.14. Switch to the FIB-SEM software and draw a lamella at the region of interest **[1]**.

2.14.1. SCREEN: 2.13.1-(1).mp4 00:00-00:10

2.15. Perform rough milling to remove the bulk material ~~approximately 3 micrometers above and below the region of interest~~ while keeping the fluorescence microscope on to monitor signal changes **[1]**.

2.15.1. SCREEN: 2.14.1-(1).mp4 00:00-00:04
and 2.14.1-(1)_complement.mp4 00:00-00:04

Video Editor: Please play the files one after the other

2.16. Open the Python-based interferometric toolkit from the terminal to monitor fluorescent signal during lamella thinning **[2]**. Thin the **ROI** *(R-O-I)* to approximately **2 µM** *(micrometers)* using the **cleaning cross-section** mode **[1]**.

2.16.1. SCREEN: 2.16and17-(1).mp4 00:02-00:06,

2.16.2. SCREEN: 2.15.1_correct-(1).mp4 01:10-01:26

2.17. For a target with an axial extent of over 300 nanometers, in the Python GUI *(G-U-I)*, select the target in the fluorescence micrograph **[1].** The software will automatically begin plotting brightness as a function of time with each new frame **[2]**.

2.17.1. SCREEN: 2.16and17-(1).mp4 00:25- 00:40

2.17.2. SCREEN: 2.16and17-(1).mp4 00:41-00:58

2.18. Monitor fluorescence intensity through the Python toolkit **[1].** Mill the sample using cleaning cross-section mode to remove any remaining support film from below the region of interest **[2-TXT]**.

2.18.1. SCREEN: 2.16and17-(1).mp4 01:00-01:14

Video Editor: Here, emphasize the panel on the right with the red curve

2.18.2. SCREEN: 2.16and17-(1).mp4 01:25-01:35

TXT: Fluorescence is enhanced when support film is removed

Video Editor: Here, emphasize the panel on the left with the red box

2.19. Observe for sudden drops in fluorescence intensity as cues to switch milling direction **[1]**. If a sharp decrease is observed when milling from the bottom up, stop and begin milling from the top down **[2-TXT]**.

2.19.1. SCREEN: 2.16and17-(1).mp4 01:35-01:42

2.19.2. SCREEN: 2.15.1_correct-(1).mp4 02:45-03:00

TXT: If structure is removed too quickly, reduce milling current to slow down target removal

2.20. After completing top-down milling, finalize the lamella by milling from the bottom up in cleaning cross-section mode until the desired thickness is reached **[1]**. Then capture a post-milling fluorescence image of the lamella **[2]**.

2.20.1. SCREEN: 2.19.1-(1).mp4 00:00-00:30

Videographer's Note: I also recorded the talent removing the rod, in case it's needed

2.20.2. SCREEN: 2.19.2_final_micrograph-(1).mp4 00:07-00:17
Fluorescence image acquisition showing the lamella post milling.

2.21. Transfer the sample to a CryoTEM *(Cry-o-Tem)* **[1]**~~. collect an atlas overview of the grid at 182 X to cover the entire grid **[2-TXT].**~~

2.21.1. Shot of the sample in a CryoTEM.

~~2.21.2. SCREEN: Atlas overview of the entire grid captured at 182 X in the CryoTEM interface. **TXT: Use Tomo5 software**~~
NOTE: Shot deleted by authors

~~2.22. Then acquire a montage series of images of the final lamella at 0 degree tilt at appropriate intermediate magnifications **[1-TXT].**~~

~~2.22.1. SCREEN: Montage imaging window displaying stitched images of the lamella at intermediate magnification and 0 degree tilt. **TXT: Magnifications: 6500X to 11,000X**~~
NOTE: Shot deleted by authors

2.23. Load the post-milling multi-channel fluorescence image and the CryoTEM projection image into the custom projective transformation software for region of interest registration **[1]**. Now select 8 to 10 pairs of reference points that are visible in both fluorescence and electron microscopy images to compute projective transformation **[2]**. Use the overlaid ~~fluorescence and transmission electron microscopy~~ image as a guide to select an imaging area ~~within the camera frame size for high magnification tilt-series collection~~ **[3].**

2.23.1. SCREEN: 2.22-(1).mp4 00:04-00:28, 00:46

2.23.2. SCREEN: 2.22-(1).mp4 01:07-01:21, 01:50-01:53,02:19-02:20
AND

SCREEN: 2.22-(1).mp4 03:08-03:12, 03:55-04:00
Video Editor: Please play both files side by side in a split screen

2.23.3. SCREEN: 2.22-(1).mp4 04:30-04:36

2.24. Then select appropriate magnification and acquisition parameters for the tilt-series, depending on the biological objective **[1]**.

2.24.1. SCREEN: 2.23.1-(1).tif

Results

3. Results

3.1. Differentiated macrophage cells labeled with SiR *(S-I-R)* -tubulin displayed a single bright fluorescent punctum approximately 1 micrometer in diameter, corresponding to the MTOC *(M-T-O-C)* **[1-TXT]**, along with fibril-like structures radiating towards the cell periphery, consistent with the microtubule network **[2]**.

3.1.1. LAB MEDIA: Figure 1A (left image) *Video editor: Highlight the single bright red dot in each cell, marked by yellow arrowheads*
TXT: MTOC : Microtubule Organizing Center

3.1.2. LAB MEDIA: Figure 1A (right image) *Video editor: Highlight the red fibril-like strands marked by yellow arrowheads*

3.2. As the sample was milled to approximately 800 nanometers in thickness, the singular fluorescent MTOC punctum resolved into two distinct speckles, each approximately 700 nanometers in diameter **[1]**.

3.2.1. LAB MEDIA: Figure 1B *Video Editor: Please sequentially show the columns "pre-milling" to "800 nm" then highlight the 800 nm column images*

3.3. Fluorescence intensity increased after removal of non-fluorescent bulk material and the absorptive support film **[1]**, followed by a sharp decrease as the lamella was further thinned from 2 micrometers to below 200 nanometers **[2]**.

3.3.1. LAB MEDIA: Figure 1 C. *Video editor: Highlight the middle region of the timeline where the curve rises, marked by the label "Enhancement due to the removal of the absorptive support film".*

3.3.2. LAB MEDIA: Figure 1D. *Video editor: Emphasize the downward trend in the red lines labeled 180 nm and the lighter red line labeled 500 nm*.

3.4. Correlated fluorescence and electron microscopy imaging enabled precise targeting of structures during milling **[1]**. Reconstructed tomograms and segmented models reveal that the centrioles are composed of microtubule triplets **[2]**.

3.4.1. LAB MEDIA: Figure 2A(a–c). *Video editor: Highlight the areas pointed at by the yellow arrows*

3.4.2. LAB MEDIA: Figure 2A (d). *Video editor: Highlight the areas pointed at by the yellow arrows*.

3.5. In cases where only one fluorescent speckle was retained in the final lamella, a single centriole was visualized in the corresponding tomogram **[1]**.

3.5.1. LAB MEDIA: Figure 2B *Video editor: Highlight the areas pointed at by the yellow arrows and the inset*

Pronunciation Guide:

1. Cryogenic
   Pronunciation link: https://www.merriam-webster.com/dictionary/cryogenic
   IPA: /ˌkraɪ.əˈdʒɛn.ɪk/
   Phonetic Spelling: kry·uh·jen·ik
2. Coincident
   Pronunciation link: https://www.merriam-webster.com/dictionary/coincident
   IPA: /koʊˈɪn.sɪ.dənt/
   Phonetic Spelling: koh·in·suh·duhnt
3. Fluorescence
   Pronunciation link: https://www.merriam-webster.com/dictionary/fluorescence
   IPA: /ˌflʊrˈɛs.əns/
   Phonetic Spelling: floor·eh·suhns
4. Electron
   Pronunciation link: https://www.merriam-webster.com/dictionary/electron
   IPA: /ɪˈlɛk.trɑn/
   Phonetic Spelling: ih·lek·tron
5. Ion
   Pronunciation link: https://www.merriam-webster.com/dictionary/ion
   IPA: /ˈaɪ.ɑn/
   Phonetic Spelling: eye·on
6. Microscope
   Pronunciation link: https://www.merriam-webster.com/dictionary/microscope
   IPA: /ˈmaɪ.krəˌskoʊp/
   Phonetic Spelling: my·kruh·skohp
7. Lamella
   Pronunciation link: https://www.merriam-webster.com/dictionary/lamella
   IPA: /ləˈmɛl.ə/
   Phonetic Spelling: luh·mel·uh
8. Autogrid
   Pronunciation link: No confirmed link found
   IPA: /ˈɔːˌtoʊˌgrɪd/
   Phonetic Spelling: aw·toh·grid
9. Cryo-FIB-SEM
   Pronunciation link: No confirmed link found
   IPA: /ˈkraɪ.oʊ fɪb sɛm/
   Phonetic Spelling: kry·oh fib sem
10. Correlative
    Pronunciation link: https://www.merriam-webster.com/dictionary/correlative
    IPA: /kəˈrɛl.ə.tɪv/
    Phonetic Spelling: kuh·rel·uh·tiv
11. Microscopy
    Pronunciation link: https://www.merriam-webster.com/dictionary/microscopy

IPA: /maɪˈkrɑs.kə.pi/
Phonetic Spelling: my·kros·kuh·pee

12. Fluorophore
Pronunciation link: https://www.merriam-webster.com/dictionary/fluorophore
IPA: /ˈflʊr.əˌfɔr/
Phonetic Spelling: floor·uh·for
13. Z-stack
Pronunciation link: No confirmed link found
IPA: /ˈziː stæk/
Phonetic Spelling: zee·stak
14. Micrometer
Pronunciation link: https://www.merriam-webster.com/dictionary/micrometer
IPA: /maɪˈkrɑm.ə.ɾɚ/
Phonetic Spelling: my·krah·muh·ter
15. Nanometer
Pronunciation link: https://www.merriam-webster.com/dictionary/nanometer
IPA: /ˈnæn.oʊˌmiː.ɾɚ/
Phonetic Spelling: nan·oh·mee·ter
16. Interferometric
Pronunciation link: https://www.merriam-webster.com/dictionary/interferometric
IPA: /ˌɪn.ɾɚ.fɪr.oʊˈmɛt.rɪk/
Phonetic Spelling: in·ter·feer·oh·met·rik
17. CryoTEM
Pronunciation link: No confirmed link found
IPA: /ˈkraɪ.oʊ tɛm/
Phonetic Spelling: kry·oh tem
18. Tomography
Pronunciation link: https://www.merriam-webster.com/dictionary/tomography
IPA: /təˈmɑg.rə.fi/
Phonetic Spelling: tuh·mog·ruh·fee
19. Tomogram
Pronunciation link: https://www.merriam-webster.com/dictionary/tomogram
IPA: /ˈtɑm.əˌgræm/
Phonetic Spelling: tom·uh·gram
20. Centriole
Pronunciation link: https://www.merriam-webster.com/dictionary/centriole
IPA: /ˈsɛn.triˌoʊl/
Phonetic Spelling: sen·tree·ohl
21. Microtubule
Pronunciation link: https://www.merriam-webster.com/dictionary/microtubule
IPA: /ˌmaɪ.kroʊˈtuː.bjuːl/
Phonetic Spelling: my·kroh·too·byool

22. Macrophage
    Pronunciation link: https://www.merriam-webster.com/dictionary/macrophage
    IPA: /ˈmæk.rəˌfeɪdʒ/
    Phonetic Spelling: mak·ruh·fayj
23. SiR-tubulin
    Pronunciation link: No confirmed link found
    IPA: /ˈɛs.aɪ.ɑr ˈtuː.bjʊ.lɪn/
    Phonetic Spelling: ess·eye·ar too·byuh·lin
24. MTOC
    Pronunciation link: No confirmed link found
    IPA: /ˌɛm.tiː.oʊˈsiː/
    Phonetic Spelling: em·tee·oh·see
25. Fluorophore
    Pronunciation link: https://www.merriam-webster.com/dictionary/fluorophore
    IPA: /ˈflʊr.əˌfɔr/
    Phonetic Spelling: floor·uh·for
26. Refractive
    Pronunciation link: https://www.merriam-webster.com/dictionary/refractive
    IPA: /rɪˈfræk.tɪv/
    Phonetic Spelling: rih·frak·tiv
27. Diffraction
    Pronunciation link: https://www.merriam-webster.com/dictionary/diffraction
    IPA: /dɪˈfræk.ʃən/
    Phonetic Spelling: dih·frak·shuhn
28. Registration
    Pronunciation link: https://www.merriam-webster.com/dictionary/registration
    IPA: /ˌrɛdʒ.ɪˈstreɪ.ʃən/
    Phonetic Spelling: rej·ih·stray·shuhn
29. Projective
    Pronunciation link: https://www.merriam-webster.com/dictionary/projective
    IPA: /prəˈdʒɛk.tɪv/
    Phonetic Spelling: pruh·jek·tiv
30. Transformation
    Pronunciation link: https://www.merriam-webster.com/dictionary/transformation
    IPA: /ˌtræns.fəˈmeɪ.ʃən/
    Phonetic Spelling: trans·fer·may·shuhn