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Title: A Scanning Electron Microscopy-Compatible Optical Imaging Method for Mesoscopic All-Cell Brain Mapping

Authors and Affiliations:

Zhongyang Li[1,2,3], Peiyao Shi[4], Ruobing Zhang[2,4]

[1]School of Biomedical Engineering (Suzhou), Division of Life Sciences and Medicine, University of Science and Technology of China
[2]Anhui Province Key Laboratory of Biomedical Imaging and Intelligent Processing, Institute of Artificial Intelligence, Hefei Comprehensive National Science Center
[3]Department of Biomedical Engineering, City University of Hong Kong, Hong Kong, China
[4]Institute for Brain and Intelligence, Fudan University

Corresponding Authors:
Ruobing Zhang rbzhang@fudan.edu.cn

Email Addresses for All Authors:
Zhongyang Li lzyyy@mail.ustc.edu.cn
Peiyao Shi shipeiyao1228@163.com
Ruobing Zhang rbzhang@fudan.edu.cn

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?

   No

   JoVE will use our scope kit.

   If your microscope does not have a camera port, the scope kit will be attached to one of the eyepieces and **you will have to perform the procedure using one eye**.

   microscope **: (AutoCUTS II (Lehua))**

   SCOPE Shots: **3.3.3, 3.6.1, 3.10.3**

   Videographer: Please film the above-mentioned shots using the scope kit

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, 5 done [2 captured by videographer (4.1.3. and 4.1.4)]**

Videographer: Please record the computer screen for the shots labeled as SCREEN as back-up

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

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: 23
Number of Shots: 52 (7 SC, 3 Scope)

Introduction

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

NOTE from Utkarsh: Interviews uploaded on 23 sep were better than what's being uploaded on 26, but if sound quality can be cleared then it might work, I will leave decision to editor whichever they find more appropriate.

1.1. **Ruobing Zhang:** Our research focuses on connectomics. We develop high-throughput optical or electron image acquisition methodologies, enabling the analysis of neural circuitry and synaptic connectivity features to decipher the fundamental connection logic of neural circuits.

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 research gap are you addressing with your protocol?

1.2. **Zhongyang Li:** Traditional high-resolution connectomics is limited to small brain volumes due to costly data acquisition and processing, whereas OMLIT enables rapid mesoscopic analysis and targeted microscopic study, offering potential for whole-brain connectomics.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.2.1*

What advantage does your protocol offer compared to other techniques?

1.3. **Zhongyang Li:** Compared to other mesoscopic approaches such as various fluorescence microscopies, OMLIT captures images of all neurons with dendrite and axon wiring information. Besides, its compatibility with electron microscopy enables further nanoscale resolution imaging under EM.

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

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

1.4. **Ruobing Zhang:** OMLIT helps build comprehensive brain models, mapping all long-range projections—including their direction, strength, and type—alongside local microcircuits, offering insights into the information flow and functional regulation across the whole brain.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll:5.1.2*

What questions will future research focus on?

1.5. **Ruobing Zhang:** We are developing automated OMLIT imaging and EM data acquisition guided by OMLIT-defined ROIs, enabling efficient connectomic data collection and analysis across large or whole brain volumes.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.5.1*

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

2. Preparation of the Tape: High- and Low-Reflectivity Strategies

Demonstrator: Peiyao Shi

2.1. To begin, select either Kapton or D-50 film with a thickness of 50 micrometers and mount it onto the motorized winding system **[1-TXT].**

2.1.1 WIDE: Talent placing polyimide tape or D-50 film onto the motorized winding system and securing it in place. **TXT: Metal coating to the tape**

2.2. Using a double-head magnetron sputtering system, deposit a uniform thin film of chromium or other metals such as aluminum, silver, or copper onto the surface of the tape **[1]**. Position the tape at a distance of 80 millimeters from the sputtering target **[2]**. Perform the process under direct current power and at a pressure of 1 pascal regulated by 99.99 percent argon gas **[3]**.

2.2.1. Talent operating the magnetron sputtering system to deposit a thin metallic coating onto the tape.

2.2.2. Close-up of the tape positioned 80 millimeters away from the sputtering target inside the chamber.

2.2.3. Show system settings indicating direct current power mode, chamber pressure at 1.0 pascal, and argon gas regulation at 99.99percent purity.

2.3. Now, set the tape winding speed to 0.6 millimeters per second to achieve a metal coating thickness of 50 nanometers **[1]**. After deposition, take out the tape from the system once it has cooled down to room temperature **[2]**.

2.3.1. Show the tape winding speed set to 0.6 millimeters per second on the sputtering system interface.

2.3.2. Shot of taking the coated tape from the chamber. **NOTE**: The action and VO were modified

2.4. Evaluate the thickness and uniformity of the coating using a stylus profiler, atomic force microscopy, or scanning electron microscopy **[1]**.

2.4.1. Talent operating a atomic force microscope, or scanning electron microscope.

2.5. For low reflectivity strategy, clean and render the tape hydrophilic using a plasma cleaner at 80-watt power while moving the tape at 7 millimeters per second **[1-TXT]**. After treatment, place water droplets on the tape surface to confirm they rapidly spread into a thin film **[2]**.

2.5.1. Talent operating the plasma cleaner with tape passing through at 7 millimeters per second under 80 watt power. **TXT: Subsequent steps are similar for both high and low reflectivity strategies**

2.5.2. Close-up of water droplets spreading rapidly into a thin film on the treated tape surface.

3. Serial Ultrathin Sectioning and Tape-Based Collection

3.1. Using a small grinder or similar cutting tool, rough-trim the resin on the sample side to remove surrounding blank resin and expose the sample area **[1]**.

3.1.1. Talent trimming away excess resin with a small grinder to reveal the embedded sample.

3.2. Place the resin-embedded block in the sample holder and tighten it to secure the block firmly **[1]**.

3.2.1. Talent placing the resin-embedded block in the sample holder and tighten it.

3.3. Mount the sample holder on the microtome's movable arm **[1]**. Install a glass knife or diamond trimming knife at a 45-degree angle on the knife holder **[2]**. Under the microscope, trim the sample surface into a pyramid shape and smooth it **[3]**.

3.3.1. Talent transferring the sample holder onto the microtome's movable arm.

3.3.2. Talent installing a trimming knife at a 45-degree angle onto the knife holder.

3.3.3. SCOPE: View of the sample surface being trimmed into a pyramid shape and smoothed.

3.4. Then, trim and smooth the four sides of the sample block to remove excess resin **[1]**. Rotate the knob to align the front and rear edges of the block in a horizontal position **[2]**.

3.4.1. Talent trimming the sides of the block to remove excess resin.

3.4.2. Close-up of talent rotating the knob to ensure horizontal alignment of the block.

3.5. Remove the trimming knife and replace it with a diamond knife set at a 45-degree angle **[1]**. Set the tilt angle of the microtome base to 6 degrees **[2]** and move the knife holder slowly using the knob until the front edge of the knife is 1 to 2 millimeters from the sample surface **[3]**.

3.5.1. Talent replacing the trimming knife with a diamond knife set at 45 degrees.

3.5.2. Show the microtome base tilt angle set to 6 degrees.

3.5.3. Close-up of the diamond knife positioned 1 to 2 millimeters from the sample surface.

3.6. Observe the bright band between the sample surface and the knife edge **[1]** and adjust the tilt angle so the band is even from top to bottom and side to side **[2]**.

3.6.1. SCOPE: Bright band visible between the sample surface and knife edge.

3.6.2. Show tilt angle adjustments being made.

3.7. Now, inject distilled water into the groove of the diamond knife to wet the blade **[1]**. Remove some water with a syringe until the liquid level dips and the reflection appears silvery **[2]**.

3.7.1. Talent injecting distilled water into the groove of the diamond knife.

3.7.2. Close-up of talent using a syringe to remove water until the reflection turns silvery.

3.8. Next, set the section thickness, cutting speed, and cutting window in the control unit **[1]**. Adjust the sectioning speed to 0.6 millimeters per second and the section thickness to 60 nanometers, depending on the sample quality **[2]**.

3.8.1. Shot of settings being entered into the control unit for section thickness, cutting speed, and cutting window.

3.9. Begin sectioning with the microtome **[1]**. Once stable and uniform sections are produced, pause the process **[2]**. Then, use a fine brush to remove cut sections and debris **[3]**.

3.9.1. Talent initiating sectioning on the microtome.

3.9.2. Close-up of uniform sections forming during stable operation.

3.9.3. Talent carefully removing sections and debris with a fine brush.

3.10. Now, install both the coated tape reel and an empty take-up reel on the automatic ultrathin section collecting system **[1]**. Secure the locking mechanism **[2]** and perform a trial run to confirm smooth tape movement at constant speed **[3]** and proper collection onto the take-up reel **[4]**.

3.10.1. Talent installing the coated tape reel and an empty reel on the collection system.

3.10.2. Close-up of talent securing the locking mechanism.

3.10.3. SCOPE: Shot of trial run of tape moving smoothly.

3.10.4. Shot of the tape winding onto the empty reel.

3.11. Immerse the collection head of the tape collection device in the diamond knife's water bath **[1]** and adjust the head to be parallel to the knife edge at 1.5 times the sample slice length **[2]**. Secure the collection device and resume sectioning while simultaneously running the tape collection device **[3]**.

3.11.1. Talent immersing the collection head into the water bath.

3.11.2. Close-up of the collection head adjusted parallel to the knife edge at the specified distance.

3.11.3. Talent resuming sectioning with the tape collection device operating simultaneously.

3.12. After collecting sufficient continuous sections, pause sectioning **[1]**. Cut the tape in an area without sections **[2]**. Run the tape collection device until all tape is wound onto the spool **[3]**.

3.12.1. Talent pausing the sectioning process.

3.12.2. Talent cutting the tape at a blank region.

3.12.3. Shot of the tape collection device running until all tape is fully collected onto the spool.

3.13. Next, remove the spool and place it in an electronic drying oven **[1]**. Clean the tape collection device and the microtome, and return all accessories to their proper places **[2]**.

3.13.1. Talent placing the spool with collected sections into an electronic drying oven.

3.13.2. Talent cleaning the tape collection device and microtome, then organizing

accessories.

3.14. For mounting on a silicon wafer, peel off the transparent protective film from the double-sided conductive tape **[1]**. Apply the tape parallel to the double-sided conductive tape **[2-TXT]**, placing up to three segments of tape on each piece **[3-TXT]**.

3.14.1. Talent peeling away the transparent protective film from the double-sided conductive tape.

3.14.2. Talent applying the tape parallel to the double-sided conductive tape. **TXT: For D-50 tape, remove the protective film on the back**

3.14.3. Close-up of up to nine tape segments neatly aligned on three pieces of double-sided conductive tape. **TXT: Process the sample after post-staining procedure**

4. Data Acquisition: Optical Microscopy

4.1. Place the silicon wafer on the stage of the optical microscope and secure it with non-residue adhesive tape **[1]**. Use a 5x objective lens to obtain an overview image of the silicon wafer, the tape, and the sample **[2]**. On the overview image, outline each section and sort them, then add focus and exposure points to perform automatic imaging at 20 or 50x magnification across the entire wafer **[3]**. After imaging, save the images and check their quality, refocusing and reimaging if any are out of focus or poor quality **[4]**.

4.1.1. Talent placing the silicon wafer on the microscope stage and securing it with non-residue adhesive tape.

4.1.2. Talent adjusting the 5x lens.

4.1.3. SCREEN: Show outlining of each section, sorting, and placement of focus and exposure points for automatic imaging at 20 times or 50 times magnification. ***Videographer: Please record the computer screen for the shots 4.1.3 and 4.1.4***

4.1.4. SCREEN: Saving image files and checking quality, followed by refocusing and reimaging poor-quality sections.

4.2. Import the TIFF *(tiff)* image stack into VAST22 *(vast-22)* by selecting **Import** followed by **Import image volume from images to .VSV** *(V-S-V)* **File [1]**.

4.2.1. SCREEN: 68814_screen_shot_3.mp4 00:07-00:20.

4.3. Connect an external tablet and use the brush tool in **Draw Segment Mode** to manually segment and trace structures **[1]**. Use the shortcut keys **A** and **Z** to navigate between image slices **[2]**.

4.3.1. SCREEN: 68814_screen_shot_4.mp4 00:05-00:07 and 00:28-00:35.

4.3.2. SCREEN: 68814_screen_shot_4.mp4 02:06-02:20.

4.4. Now, visualize the segmented structure in three dimensions by selecting **Window** followed by **3D Viewer**, **View** and **Update [1]**. Finally, save the segmentation results after selecting **File** and **Save Segmentation [2]**.

4.4.1. SCREEN: 68814_screen_shot_5.mp4 00:05-00:20.

4.4.2. SCREEN: 68814_screen_shot_5.mp4 00:46-00:58.

Results

5. Results

5.1. In the high-reflectivity images, the cytoplasmic and vascular lumen regions showed higher intensity compared to the surrounding areas **[1]**, while in the low-reflectivity images, the cytoplasmic and vascular lumen regions exhibited lower intensity **[2]**.

5.1.1. LAB MEDIA: Figure 3A B C.

5.1.2. LAB MEDIA: Figure 3D E F.

5.2. Quantified results demonstrated that the high-reflectivity and low-reflectivity strategies each had advantages in terms of contrast and information entropy **[1]**.

5.2.1. LAB MEDIA: Table 1. *Video editor: Highlight columns "contrast" and "information entropy".*

5.3. OMLIT *(O-M-lit)* optical microscopy imaging allowed identification of axons **[1-TXT]**, blood vessels **[2]**, cell bodies **[3]**, and dendrites **[4]**.

5.3.1. LAB MEDIA: Figure 4A. Video editor: Highlight the blue arrow marking axons. **TXT: OMLIT: Optical Multilayer Interference Tomography**

5.3.2. LAB MEDIA: Figure 4A. *Video editor: Highlight the green arrow marking blood vessels.*

5.3.3. LAB MEDIA: Figure 4A. *Video editor: Highlight the yellow arrow marking cell bodies.*

5.3.4. LAB MEDIA: Figure 4A. *Video editor: Highlight the orange arrow marking dendrites.*

5.4. Manual segmentation of OMLIT images using VAST showed numerous tightly arranged cell bodies, dendrites, and axons **[1]**.

5.4.1. LAB MEDIA: Figure 4B. *Video editor: Highlight the segmented tracing where many cell bodies, dendrites, and axons appear close together.*

5.5. The segmented results were combined with the original image to produce a three-dimensional visualization **[1]**.

5.5.1. LAB MEDIA: Figure 4C. *Video editor: Show the overlay of the segmented tracing with the original microscopy image.*

5.6. OMLIT imaging displayed a prominent blood vessel that served as a landmark in the same region as electron microscopy **[1]**.

5.6.1. LAB MEDIA: Figure 5A. *Video editor: Highlight the white arrow pointing to the blood vessel.*

5.7. Magnified OMLIT images showed insufficient resolution to reveal finer structures **[1]**.

5.7.1. LAB MEDIA: Figure 5B–C. *Video editor: Show the zoomed-in views of the OMLIT image where details remain blurred.*

5.8. Electron microscopy images of the same region revealed synapses, mitochondria, cell nuclei, and vesicles **[1]**.

5.8.1. LAB MEDIA: Figure 5D–E.

- **Kapton**

Pronunciation link:
https://www.merriam-webster.com/dictionary/Kapton
IPA: /ˈkæp.tɒn/
Phonetic Spelling: kap-ton

- **Micrometers**

Pronunciation link:
https://www.merriam-webster.com/dictionary/micrometer
IPA: /maɪˈkrɑːmɪtɚz/ (as in "millionth of a meter")
Phonetic Spelling: my-krah-muh-terz

- **Polyimide**

Pronunciation link:
https://www.merriam-webster.com/dictionary/polyimide
IPA: /ˌpɑːliˈɪmaɪd/
Phonetic Spelling: pah-lee-ih-mide

- **Magnetron**

Pronunciation link:
https://www.merriam-webster.com/dictionary/magnetron
IPA: /ˈmæg.nəˌtrɑːn/
Phonetic Spelling: mag-nuh-tron

- **Sputtering**

Pronunciation link:
https://www.merriam-webster.com/dictionary/sputtering
IPA: /ˈspʌt̬.ɚ.ɪŋ/
Phonetic Spelling: sputt-er-ing

- **Chromium**

Pronunciation link:
https://www.merriam-webster.com/dictionary/chromium
IPA: /ˈkroʊ.mi.əm/
Phonetic Spelling: kroh-mee-uhm

- **Nanometers**

Pronunciation link:
https://www.merriam-webster.com/dictionary/nanometer
IPA: /ˈnæn.oʊˌmiːt̬ɚz/
Phonetic Spelling: nan-oh-mee-terz

- **Stylus**

Pronunciation link:
https://www.merriam-webster.com/dictionary/stylus
IPA: /ˈstaɪ.ləs/
Phonetic Spelling: sty-lus

- **Profiler**

Pronunciation link:
https://www.merriam-webster.com/dictionary/profiler
IPA: /ˈproʊˌfaɪ.lɚ/
Phonetic Spelling: proh-fy-ler

- **Microscopy**

Pronunciation link:
https://www.merriam-webster.com/dictionary/microscopy
IPA: /maɪˈkrɑː.skə.pi/
Phonetic Spelling: my-krah-skuh-pee

- **Hydrophilic**

Pronunciation link:
https://www.merriam-webster.com/dictionary/hydrophilic
IPA: /ˌhaɪ.drəˈfɪl.ɪk/
Phonetic Spelling: hy-droh-fil-ik

- **Plasma**

Pronunciation link:
https://www.merriam-webster.com/dictionary/plasma
IPA: /ˈplæz.mə/
Phonetic Spelling: plaz-muh

- **Microtome**

Pronunciation link:
https://www.merriam-webster.com/dictionary/microtome

IPA: /ˈmaɪ.krəˌtoʊm/
Phonetic Spelling: my-kroh-tome

- **Pyramid**

Pronunciation link:
https://www.merriam-webster.com/dictionary/pyramid
IPA: /ˈpɪr.ə.mɪd/
Phonetic Spelling: peer-uh-mid

- **Diamond**

Pronunciation link:
https://www.merriam-webster.com/dictionary/diamond
IPA: /ˈdaɪ.mənd/
Phonetic Spelling: dye-muhnd

- **Distilled**

Pronunciation link:
https://www.merriam-webster.com/dictionary/distilled
IPA: /dɪˈstɪld/
Phonetic Spelling: dih-stild

- **Silicon**

Pronunciation link:
https://www.merriam-webster.com/dictionary/silicon
IPA: /ˈsɪl.ɪ.kən/
Phonetic Spelling: sil-ih-kuhn

- **Optical**

Pronunciation link:
https://www.merriam-webster.com/dictionary/optical
IPA: /ˈɑːp.tɪ.kəl/
Phonetic Spelling: op-tih-kuhl

- **Magnification**

Pronunciation link:
https://www.merriam-webster.com/dictionary/magnification
IPA: /ˌmæg.nə.fəˈkeɪ.ʃən/
Phonetic Spelling: mag-nuh-fuh-kay-shun

- **VAST**

(No confirmed link found – acronym-based software)
IPA: /væst/
Phonetic Spelling: vast

- **Entropy**

Pronunciation link:
https://www.merriam-webster.com/dictionary/entropy
IPA: /ˈen.trə.pi/
Phonetic Spelling: en-truh-pee

- **OMLIT (Optical Multilayer Interference Tomography)**

No confirmed dictionary entry – acronym expansion.
IPA (OMLIT): /ˈoʊm.lɪt/
Phonetic Spelling: ohm-lit