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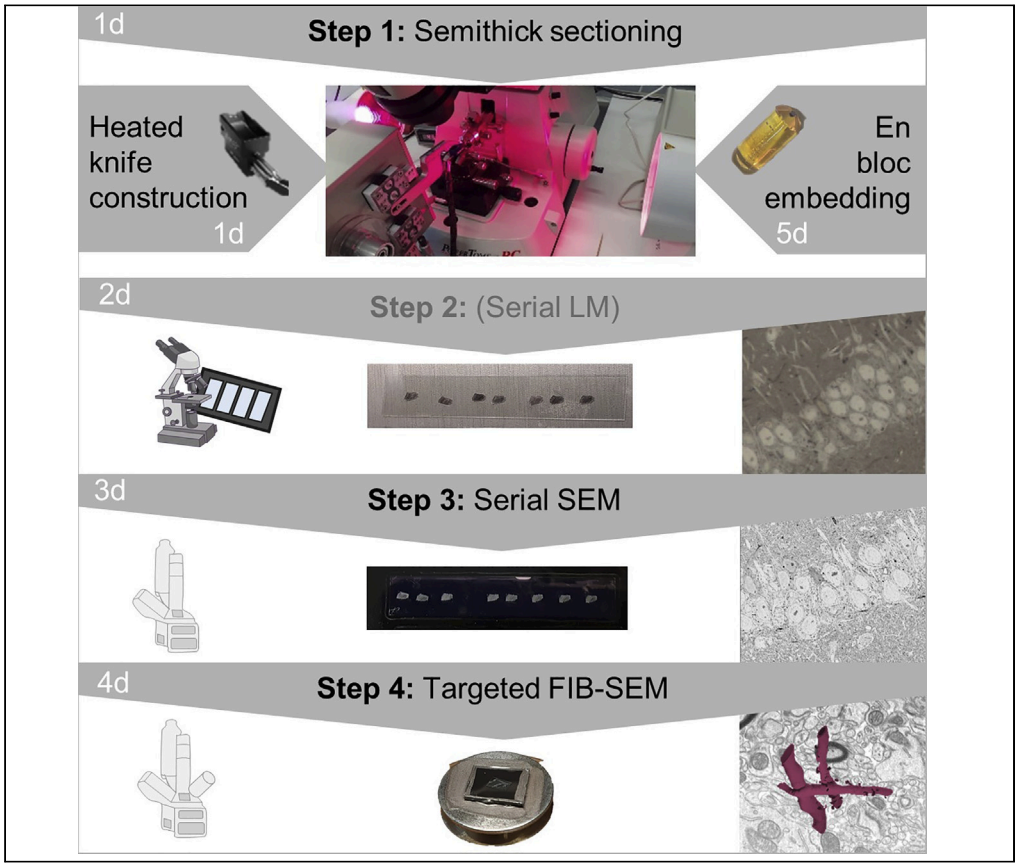
Protocol

ATUM-FIB microscopy for targeting and multiscale imaging of rare events in mouse cortex



Here, we describe a detailed workflow for ATUM-FIB microscopy, a hybrid method that combines serial-sectioning scanning electron microscopy (SEM) with focused ion beam SEM (FIB-SEM). This detailed protocol is optimized for mouse cortex samples. The main processing steps include the generation of semi-thick sections from sequentially cured resin blocks using a heated microtomy approach. We demonstrate the different imaging modalities, including serial light and electron microscopy for target recognition and FIB-SEM for isotropic imaging of regions of interest.

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HIGHLIGHTS

A protocol for serial semi-thick ultramicrotomy of resin-embedded neuronal tissue

Serial semi-thick sections on tape inspected by light and scanning electron microscopy

Targeting regions of interest by FIB-SEM with high resolution isotropic voxels

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ATUM-FIB microscopy for targeting and multiscale imaging of rare events in mouse cortex

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SUMMARY

Here, we describe a detailed workflow for ATUM-FIB microscopy, a hybrid method that combines serial-sectioning scanning electron microscopy (SEM) with focused ion beam SEM (FIB-SEM). This detailed protocol is optimized for mouse cortex samples. The main processing steps include the generation of semi-thick sections from sequentially cured resin blocks using a heated microtomy approach. We demonstrate the different imaging modalities, including serial light and electron microscopy for target recognition and FIB-SEM for isotropic imaging of regions of interest.

For complete details on the use and execution of this protocol, please refer to Kislinger et al. (2020).

BEFORE YOU BEGIN

Construction of the heated diamond knife

Timing: 1 day

1. Use a diamond knife, e.g., Diatome 35° and 45° ultra knife boats
2. Drill holes of 6 mm (lower) and 3 mm (upper) diameter at 12 mm depth into the front side of the knife boat (Figure 1).

   Note: Be careful not to destroy the diamond edge during the drilling. It is recommended to prepare the boat without the diamond and ask your knife company to insert it afterwards. Ask your diamond knife manufacturer to drill holes in the boat as shown.

3. Fit the heating parts into the knife boat
   a. Insert a sensor (cable probe 3 × 30 mm, Sensorshop24) into the upper hole
   b. Insert a heater (Hotend Heater Cartridge CNC for 3D printer, 24 V, 40 W; eBay) into the lower hole
   c. Isolate the wires using tape.

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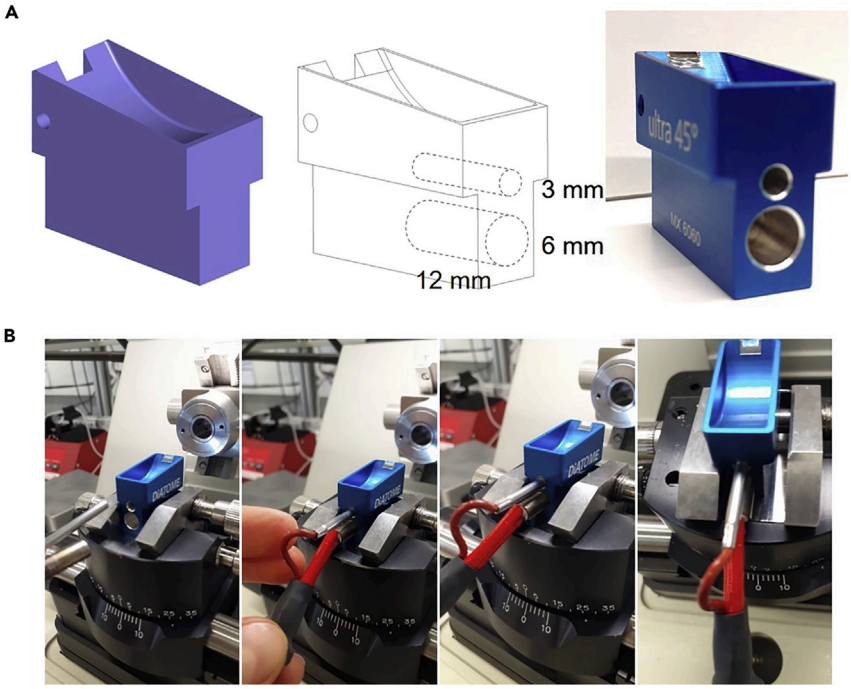

Figure 1. Heated knife construction and installation into the microtome
(A) Construction scheme and photo (right) of the knife tub including the 3 and 6 mm drillings (middle) for the sensor and heater elements, respectively. Adapted from Figure S2, Kislinger et al. (2020).
(B) Photographs of heater and sensor element insertion into the knife boat.

Note: Drill slightly bigger holes to be able to reversibly insert sensor and heater to be able to use the same heating unit for different knives. You can add conductive paste if needed. The heater and sensor can be secured but it is recommended to keep the modular nature. Heat transfer can possibly be increased by thermally conductive paste.

4. Purchase a digital on/off temperature regulator (for PT100, Sensorhop24).
5. Program the temperature regulator according to the manufacturer's manual.

Tissue fixation

Timing: 2.5 days, 2 days incubation

6. Fixative preparation

Mix 2.5 mL 16% PFA, 1 mL 25% glutaraldehyde, 10 μL 2 M calcium chloride, 2.5 mL 0.4 M sodium cacodylate buffer and 3.99 mL filtered water.

⚠ CRITICAL: Formaldehyde and cacodylate should be handled in a fume hood while wearing protective gloves and a lab coat.

Note: Prepare fixative freshly max. 2 h in advance and open a new ampoule each time.

Note: Sodium cacodylate buffer contains arsenic that contributes to the inhibition of enzymes and is therefore more effective than PBS. However, if you have safety concerns the cacodylate buffer can be replaced by 0.1 M PBS.

7. Perfusion
   a. Adjust the flow rate to ~ 1.3 mL/min.
   b. Wash with one volume (~6 mL per adult mouse) HBSS (if available with heparin).
   c. Switch to fixative (avoid air bubbles) immediately, continue for 15–20 min (observe muscle contraction at neck region).
   d. Carefully prepare the brain tissue and immerse it into 10–20 mL fixative in a 50 mL tube for 5 min, replace the fixative and incubate 15 h at 4°C.

⚠ CRITICAL: Prepare the tissue very carefully and avoid any stretching, distortion, drying, or squeezing which might affect the ultrastructural quality.

8. Sectioning
   a. Generate coronal sections of 0.5–1.5 mm thickness using a sharp scalpel.
   b. Immersion fix the tissue in a 15 mL tube in 5 mL fixative 24 h.
   c. Transfer the section into a 2 mL tube completely filled with 0.1 M cacodylate buffer for storage at 4°C.

Note: The thickness will depend on the specific scientific question. Vibratome slicing is possible as well. ATUM-FIB is generally suited for larger tissue volumes, so usually vibratome sections are not required but can improve staining efficiency. The smaller the tissue piece (max. 1.5 mm) the better the stain penetration in the embedding steps.

Pause Point: several days to weeks

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Cable sensor	SensorShop24	Cat# 003-KS-PT100-2L-1.0-330-W
Carbon adhesive tape	Science Services	Cat# P77819-25
Carbon nanotube tape (CNT TPEN, Typ TEI, 8 mm wide)	Science Services	Cat# R-ATUM313
Controller for PT100	SensorShop24	Cat# TR-PT100-A-24V
Diamond knife 35°, 45°, 3 mm	Diatome/Science Services	Cat# DU3530; DU4530
Diamond trimming knife trim 90	Diatome/Science Services	Cat# DTB90
FIB-SEM (e.g., Crossbeam 340)	Zeiss	N/A
Glass vial with rolled rim, short (5 mL), with lid	Carl Roth	Cat# CLA1.1
Heating cartridge, 24 V, 40 W	eBay	N/A
Laboratory microwave (e.g., Pelco Biowave Pro), with vacuum chamber and temperature control (SteadyTemp)	Ted Pella	Cat# 36500, 50062
Pannoramic MIDI II 2.0.5 slide scanner	3D Histech	N/A
Ultramicrotome equipped with an ATUMtome (e.g., Powertome)	RMC	N/A
Silicon wafer, 4 inch, 1 side polished, p-type (Boron), 1-0 Ohm cm	MicroChemicals	Cat# WSM40525200P1334SNN1
Standard oven (e.g., Incu-line IL 10)	VWR	Cat# 390-0384P
Chemicals, peptides, and recombinant proteins		

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Epon (Glycid ether 100, MNA, DDSA, DMP-30)	Serva	Cat# 21045.02, 29452.05, 20755.02, 36975.01,
HBSS	Sigma	Cat# H9269
Heparin	Sigma	Cat# H3393
Leit-C	Plano	Cat# G3300
Leitsilber, Acheson 1415	Plano	Cat# G3692
LX 112 Embedding Kit (LX 112 resin, DDSA, NMA, DMP 30)	LADD Research Laboratories	Cat# 21210
Osmium tetroxide	Science Services	Cat# E19130
Paraformaldehyde	Science Services	Cat# E15713
Potassium hexacyanoferrate(II) trihydrate	Sigma	Cat# 455989
Sodium cacodylate trihydrate	Science Services	Cat# E12300
Specimen mount	Science Services	Cat# 7536-30
Thiocarbohydrazide	Sigma-Aldrich	Cat# 223220
Uranyl acetate	Science Services	Cat# E22400-05G
Glutaraldehyde	Science Services	Cat# E16216
Experimental models: organisms/strains		
5xFAD (five human familial AD gene mutations) mice, 6–12 months	MMRRC	Stock# 34840-JAX/5XFAD
Wild-type mice, 6–12 months	The Jackson Laboratory	C57BL/6J
Software and algorithms		
ATLAS 5, Array Tomography, 3D	Fibics Incorporated, Canada	N/A
Blender	The Blender Foundation	https://www.blender.org/
ImageJ 2.0.0 (Fiji), TrakEM2	NIH	https://imagej.nih.gov/ij/; RRID:SCR_003070
Pannoramic software CaseViewer2.2	3D Histech	N/A
SmartSEM	Carl Zeiss Microscopy GmbH	N/A
VAST	Harvard	https://software.rc.fas.harvard.edu/lichtman/vast/

MATERIALS AND EQUIPMENT

- 0.4 M cacodylate buffer: Dissolve 42.8 g sodium cacodylate trihydrate in 300 mL water. Adjust the pH to 7.4 and fill with water up to 500 mL.

  ⚠ CRITICAL: Cacodylate contains arsenic and should be handled in a fume hood while wearing protective gloves and a lab coat.

  Note: Alternatively, a commercial buffer can be purchased (EMS). The buffer can be stored at 4°C for months.

- Fixative: Mix 2.5 mL 16% PFA, 1 mL 25% glutaraldehyde, 10 μL 2 M calcium chloride, 2.5 mL 0.4 M sodium cacodylate buffer and 3.99 mL filtered water.

Reagent	Final concentration	Amount
16% PFA	4%	2.5 mL
25% glutaraldehyde	2.5%	1 mL
2 M calcium chloride	2 mM	10 μL
0.4 M sodium cacodylate buffer	0.1 M	2.5 mL
Filtered water		3.99 mL

⚠ CRITICAL: Formaldehyde and cacodylate should be handled in a fume hood while wearing protective gloves and a lab coat.

Note: Prepare fixative freshly max. 2 h in advance and open a new ampoule each time.

- Reduced osmium tetroxide in cacodylate buffer.

mix 5 mL 4% osmium tetroxide in water with 0.25 g potassium hexacyanoferrate 2.5 mL 0.4 M sodium cacodylate buffer and 2.5 mL water

Reagent	Final concentration	Amount
Osmium tetroxide	2%	0.2 g
Potassium hexacyanoferrate	2.5%	0.25 g
0.4 M sodium cacodylate buffer	0.1 M	2.5 mL
Filtered water		7.5 mL

⚠ CRITICAL: Osmium, especially in crystalline form, is highly toxic and should be handled in a fume hood while wearing protective gloves and a lab coat.

Note: If no osmium solution but osmium crystals are used, dissolution can be accelerated by incubation in an ultrasonic bath.

Note: Potassium ferrocyanide concentration can be varied according to the tissue volume and the extent of extraction needed. If less extraction is wanted use potassium ferricyanide.

Note: Prepare this solution on the same day.

- 1% thiocarbohydrazide (TCH): Dissolve 1 g TCH in 10 mL water by stirring for 20–30 min at 60°C and filtered.

⚠ CRITICAL: TCH should be handled in a fume hood while wearing protective gloves and a lab coat.

Note: Prepare the solution freshly max. 60 min in advance.

- 2% osmium tetroxide in water: mix 5 mL 4% osmium tetroxide in water with 5 mL water.

⚠ CRITICAL: Osmium, especially in crystalline form, are highly toxic and should be handled in a fume hood while wearing protective gloves and a lab coat.

Note: Prepare this solution on the same day.

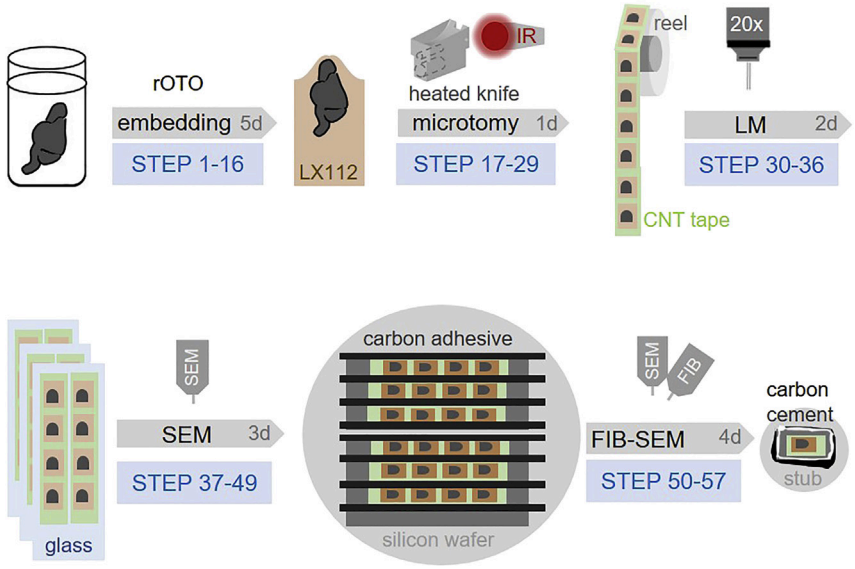

Figure 2. Schematic of sample preparation for ATUM-FIB

Coronal sections (0.5–1.5 mm thickness) are subjected to en bloc rOTO contrasting and embedded in LX112 epoxy resin. Heated ultramicrotomy facilitates (serial) semi-thick sections that are conveyed onto conductive CNT tape (green). Short tape strips bearing serial sections are mounted onto glass slides (blue) for transmitted light microscopy using a slice scanner. Remounting onto a silicon wafer with adhesive carbon tape and attachment of conductive tape strips for grounding enables subsequent surface imaging by SEM. Target sections are selected and remounted onto a stub for FIB-SEM investigation.

- 1% uranyl acetate in water: Mix 1 mL 4% uranyl acetate in water with 3 mL water. Filter.

  ⚠ CRITICAL: Uranyl acetate is a radioactive substance and highly toxic. It should be handled according to the radiation safety requirements.

  Note: The solution can be stored at 4°C for up to 6 months.

- Graded ethanol series: mix 100% ethanol with the respective amounts of water.

  Note: Store ethanol at 4°C. Opened bottles of 100% ethanol can be aliquoted and stored water-free on molecular sieves.

  Note: The dilutions can be stored at 4°C for years.

- LX112 resin: Mix 10 g of LX112 with 6.45 g of DDSA and 4.35 g of NMA and stir for 10 min. Add 0.3 mL DMP-30 and stir for 20 min.

  ⚠ CRITICAL: Uncured resin and resin components are carcinogenic and should be handled in a fume hood while wearing protective gloves and a lab coat.

  Note: Prepare the resin freshly on the same day and keep it at 20°C–24°C.

STEP-BY-STEP METHOD DETAILS

In this protocol we describe all steps of the ATUM-FIB technique from tissue preparation to FIB-SEM imaging (Figure 2).

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Table 1. *En bloc* embedding protocol

Reagent	Temperature	Microwave protocol			Bench protocol
		Time	Vacuum (in-chamber pump at 20 inHg)	Power (30 s on-30 s off-30 s on)	Time
0.1 M cacodylate buffer	20°C–24°C	3 × 10 min	off	250 W	3 × 10 min
2% osmium tetroxide, 2.5% potassium hexacyanoferrate in 0.1 M cacodylate buffer	20°C–24°C	6 × 1 min	on	300 W	2 h
	20°C–24°C	1 h (shaker)	–	–	
0.1 M cacodylate buffer	20°C–24°C	2 × 10 min	off	250 W	2 × 10 min
Water	20°C–24°C	1 min	on	250 W	10 min
1% TCH	40°C	25 min	on	200 W	45 min
Water	20°C–24°C	3 × 10 min	on	–	3 × 10 min
2% osmium tetroxide in water	20°C–24°C	6 × 1 min	on	300 W	2 h
	20°C–24°C	1 h (shaker)	–	–	
Water	20°C–24°C	3 × 10 min	on	–	3 × 10 min
1% uranyl acetate in water	4°C	15 h	–	–	15 h
	50°C	2 h	–	–	2 h
Water	20°C–24°C	1 min	on	250 W	10 min
15%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 100%, 100% ethanol	20°C–24°C	1 min each	off	200 W	10 min each
25%, 50%, 75%, 90% LX112	20°C–24°C	10 min	on	250 W	2 h
	20°C–24°C	1 h	–	–	
100% LX112	20°C–24°C	10 min	on	250 W	15 h
	20°C–24°C	15 h	–	–	
100% LX112	20°C–24°C	10 min	on	250 W	5 h
	20°C–24°C	3 h	–	–	
Curing	60°C	10, 48 h			10, 48 h

Two alternative protocols for the Tapia rOTO contrasting without lead aspartate treatment (Tapia et al., 2012) are listed and can be chosen according to the availability of a laboratory microwave.

Tissue embedding

Timing: 4 days; 10 h curing

This step describes the *en bloc* staining and embedding into resin that can be partitioned into semi-thick sections (Table 1). It is a variation of the standard rOTO (reduced osmium thiocarbohydrazide osmium) protocol (Tapia et al., 2012, Hua et al., 2015) omitting the final lead aspartate step (Figure 3).

Note: We recommend the laboratory microwave protocol both for time and quality reasons. If no microwave is available the bench protocol works as well.

1. Transfer tissue samples into a glass vials filled with 2–3 mL 0.1 M cacodylate buffer.

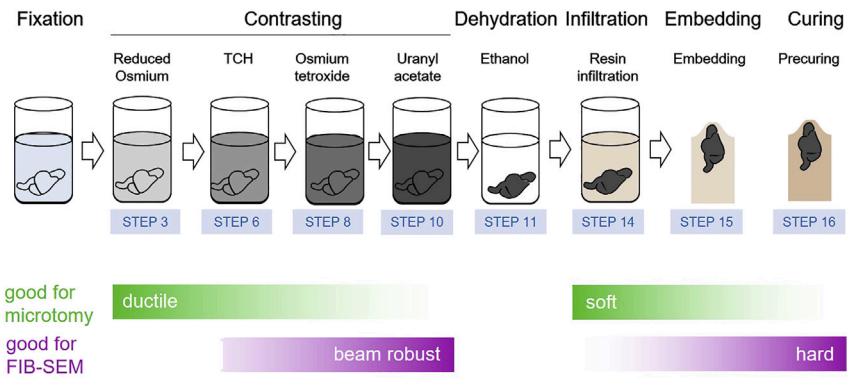

Figure 3. Scheme of the *en bloc* embedding procedure

The sequence of handling steps including tissue fixation, contrasting, dehydration, infiltration, embedding, and curing is sketched. Microtomy and FIB-SEM have conflicting requirements regarding heavy metal staining intensity and resin formulation that need to be balanced for ATUM-FIB.

Note: Reagent exchanges are performed without drying the sample at any point.

Note: Carry out incubation steps on an orbital shaker unless otherwise noted.

2. Wash the tissue at least three times with 0.1 M cacodylate buffer.

Note: If PBS was used for the perfusion, it is especially important to do thorough washing.

3. The buffer is replaced by reduced 2% osmium tetroxide in 0.1 M cacodylate buffer pH 7.4
4. Without any additional washing step, replace osmium by filtered 2.5% potassium hexacyanoferrate in 0.1 M cacodylate buffer.
5. The tissue is washed at least three times with 0.1 M cacodylate buffer.
6. Incubate in filtered 1% aqueous TCH.

Note: The TCH solution should be slightly brown but not opaque.

7. Wash at least three times with water.
8. Incubate in 2% aqueous osmium tetroxide.
9. Wash at least three times with water.
10. Transfer tissue into a fresh glass vial with 1% uranyl acetate and incubate at 4°C 15 h and 2 h at 50°C.

Note: Protect samples from light. Uranyl acetate is light sensitive and forms precipitates if exposed to UV light.

11. Dehydrate tissue in increasing ethanol concentrations (15%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 100%, 100% ethanol) by exchanges every 10–15 min.

Note: Slow dehydration is crucial for good ultrastructural quality.

Pause Point: Store samples at max. 60% ethanol 24 h if absolutely required. However, we recommend preceding immediately.

12. Incubate tissue in 100% acetone for 15 min. Repeat three times.
13. Prepare LX112 resin (Ladd Research Industries):
    a. Mix 10 g of LX112 with 6.45 g of DDSA and 4.35 g of NMA and stir for 10 min.

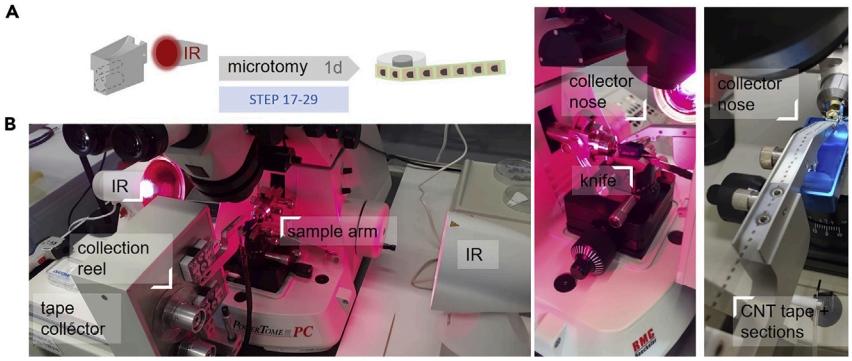

Figure 4. Heated knife semi-thick sections

(A) Schematic of heated ATUM.

(B) Ultramicrotome photos showing the heated knife, infrared lights, tape collector with nose, sample arm, and serial sections on CNT tape. Higher magnification pictures are shown as views from the left side (middle) and as a top view onto the tape collector nose capturing serial sections on CNT tape (right). Figure reprinted with permission from Kislinger et al. (2020).

b. Add 0.3 mL of DMP-30 and stir for 20 min.

14. Infiltrate tissue in LX112 resin: at least 2 h at different resin in acetone concentrations (25%, 50%, 75%, 90%) and 15 h and for another 4 h in freshly prepared 100% LX112.

Note: Resin has to be prepared freshly every day. We do not recommend freezing and thawing resins.

15. Transfer tissue onto parafilm-coated glass slides. Fill a plastic capsule with resin and invert it onto the tissue piece.
16. Cure resin blocks at 60°C for 10 h. The blocks are solid and should not be sticky anymore.

Pause Point: infinite

Generation of semi-thick sections

Timing: 2–8 h

This step describes the heated ultramicrotomy procedure that is applied to generate semi-thick sections (Figure 4).

17. Fix the cured resin block in a sample holder for trimming.
18. Use a Trim 90 knife to trim 400–800 μm at all 4 sides at a 90°C angle.

Note: Trim at full speed but at a maximum thickness of 300 nm as this ensures smooth edges and good sectioning behavior. Remove resin chips immediately using a deduster.

19. Incubate the sample holder in the oven at 40°C.
20. Install the infrared lights left and right of the diamond knife. Measure the temperature there and adjust it by varying the distance to the knife boat.
21. Fix the sample holder in the ultramicrotome.
22. Align the heated diamond knife to the sample block face.
23. Start sectioning 100–300 nm sections until the full block face appears.
24. Heat the knife to 40°C.

Note: Be careful not to touch the heater as it can get hot if it is not completely inserted into the knife boat.

25. Install the CNT (carbon nanotube) tape into the Powertome. The tension target value is 2.7–2.8. Adjust the tension by running jog forward and backward.

Optional: The CNT tape is glow-discharged by the vendor (e.g., Science Services or EMS) and can be stored several month. Hydrophilicity will gradually decrease and you can simply test it by adding a drop of water onto the surface. The better it spreads the more hydrophilic the tape surface. We recommend to freshly plasma treat the tape for better adherence of the sections especially after long-term storage over months.

26. Insert the tape nose into the water bath and approach it to a distance of approximately 1–1.5 section lengths away from the diamond edge.
27. Adjust the cutting thickness to 0.5–10 μm.

Note: Thicker sections contain more information for subsequent FIB-SEM analysis but the sectioning characteristics are more complicated to control. Uneven thicknesses, cracks, and surface heterogeneities are more often observed in sections thicker than 5 μm.

28. Start sectioning at 0.1–0.3 mm/s. Adjust the tape speed to 0.2–0.4 mm/s during cutting and to 0 during retraction. If needed guide the section onto the tape with a brush.
29. Collect serial sections and control the proper section uptake by the tape.

Note: It is recommended to stop cutting at any time and let the section flatten in the heated water bath for some seconds. Increase the cutting window if more time is needed for section uptake onto the tape.

Pause Point: months

Serial light microscopy (optional)

Timing: 1–2 days

Light microscopy of serial sections described in this paragraph (Figure 5; Methods Video S1) is optional. This depends on the dimensions of the biological object of interest or corresponding landmarks and their visibility (e.g., blood vessels, cell bodies) in transmitted light microscopy. Semi-thick sections are heavily contrasted by the rOTO protocol itself and do not require histological poststaining methods in contrast to semithin (200–500 nm) sections. If no light microscopy is intended, CNT tape can be replaced by coated (conductive) Kapton tape.

30. Disassemble the tape from the tape collector reel.
31. Cut tape with sections into 5 cm strips and keep the orientation and order.
32. Position glass slides on a heating plate (60°C).
33. Place water drops onto the glass slide.
34. Position two 5 cm tape strips per slide onto the water drops using forceps. Do not touch the sections.

Note: For the autofocus function of the slide scanner, it is crucial to get all sections into a similar plane, which is ensured by the water attachment method.

Note: If necessary, the tape strips can be additionally fixed by standard Sellotape.

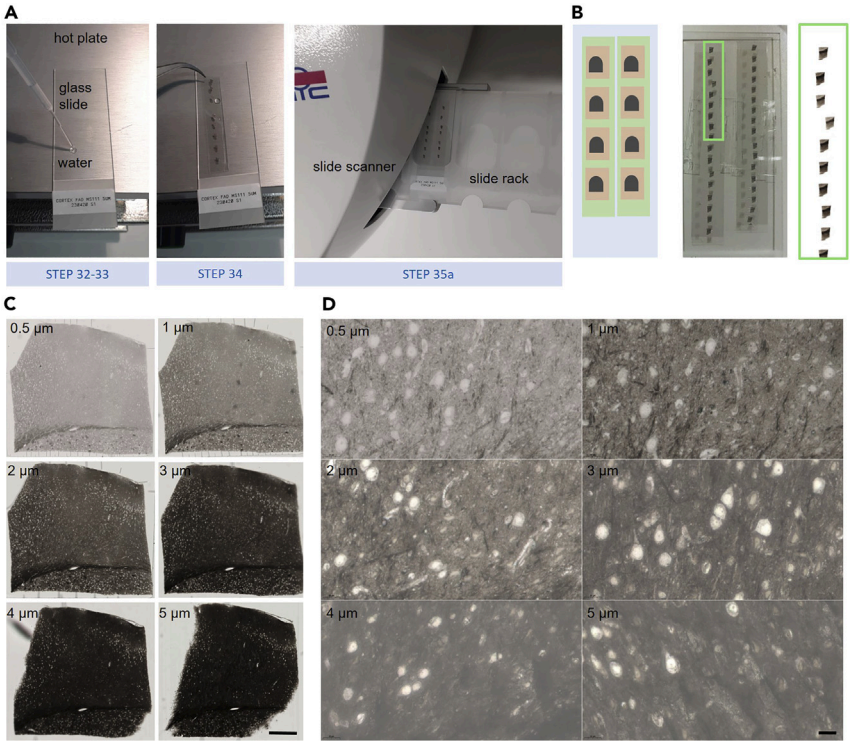

Figure 5. Transmitted light imaging

(A) Workflow of section mounting onto glass slides (left, middle) and insertion into the slide scanning microscope (right). Adapted from Figure S3, Kislinger et al. (2020).

(B) Glass slide architecture including a scheme (left), photo (middle) and transmitted light imaging results (right).

(C and D) Typical outcome for light microscopy of 0.5–5 µm thick sections showing a section overview (C) and higher magnification images (D). Scale bars, 100 µm (C), 10 µm (D).

35. Transmitted light microscopy (Pannoramic Midi II)
    a. Load the series of glass slides into a slide scanner rack.
    b. In the Pannoramic Midi II software load the first slide and manually or automatically label areas covering the tissue sections. Select sections by thresholding and image using the autofocusing and the extended focus level functions (9 focus levels, focus step size 0.2 µm × 5) using the 20× objectives. Set the autofocus range by testing it for several sections on different slide locations.
    c. Save the protocol for the respective slide and repeat step b. for all slides.
    d. Start the automated acquisition of all slides.

Note: Depending on the structures of interest either the 20× or the 40× objective can be used.

36. Generate jpeg files of the individual sections manually from the original data using the Pannoramic software CaseViewer2.2 (3D Histech).

Note: Steps 35 and 36 are exemplified for the given slide scanner but are highly dependent on the type of scanner available.

Pause Point: months

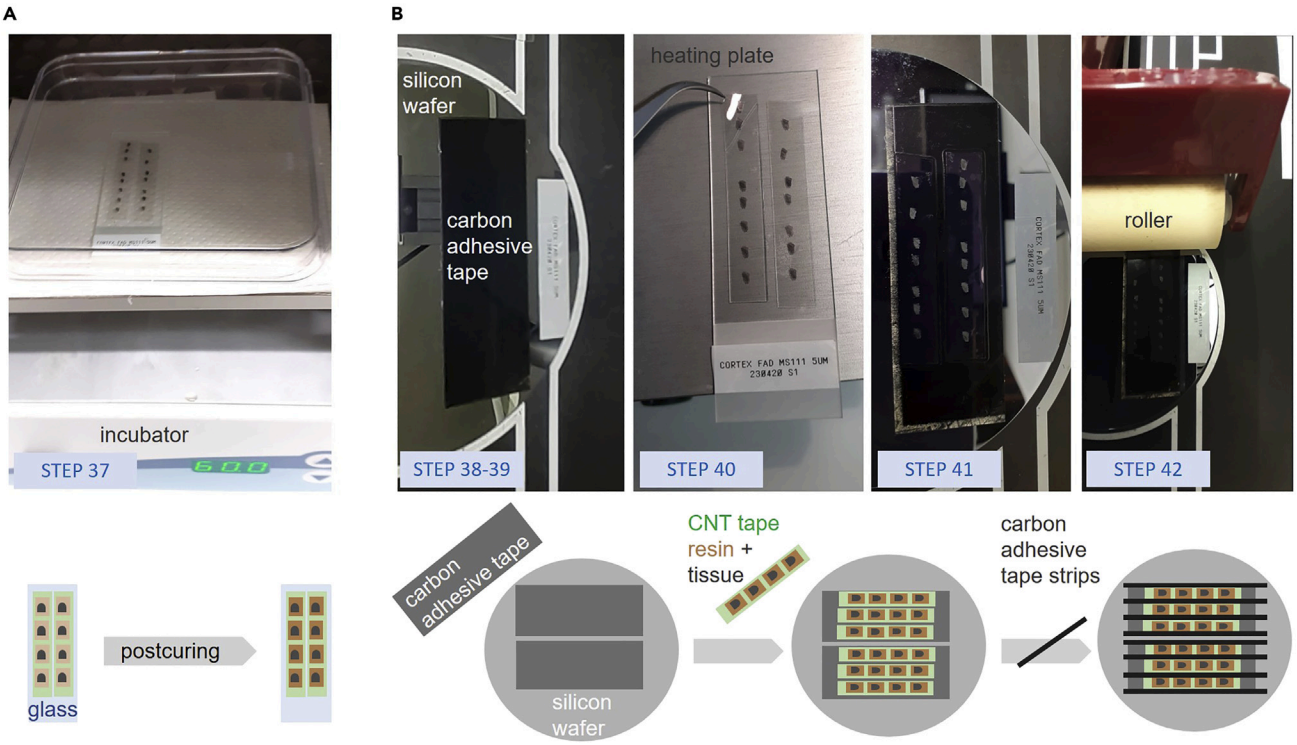

Figure 6. Silicon wafer assembly for serial SEM

(A) Photograph (top) and schematic (bottom) of a light microscopy sample postcured in an incubator at 60°C.

(B) Mounting sections requires (max. three 2.5 mm wide) carbon adhesive tape strips that are glued onto a 4 inch silicon wafer (left). The covering transparent foil is removed. Using two forceps the CNT tape strips are detached from the glass slide (middle) and positioned onto the carbon tape. Once all (max. three) strips are attached to the carbon adhesive (right top) the foil is positioned on top and the sections are flattened using a roller (right bottom). For grounding narrow carbon adhesive strips are attached to connect CNT- and carbon tape with the silicon support. The schematic is shown at the bottom.

Serial scanning electron microscopy

Timing: 1–3 days

In this section, we list the relevant steps for serial scanning electron microscopy of serial semi-thick sections (Figure 6; Methods Video S1).

37. Postcure sections on glass slides in an oven for 30–48 h at 60°C.
38. Prepare a 4-inch silicon wafer by attaching up to three conductive carbon adhesive strips (25 mm width, Science Services) onto it.
39. Remove the plastic covering the adhesive tapes and keep it.
40. Detach CNT strips with tissue sections from the glass slide using forceps.
41. Assemble CNT strips onto the adhesive tape. Be cautious not to introduce folds.
42. Cover the sections with the plastic foil kept from step 39 and smooth down the sections by hand or using a roller.

⚠ CRITICAL: The sections have to be in the same plane for the SEM autofocusing. Avoid pushing sections down too heavily to reduce the risk of introducing artifacts, especially for sections thicker than 5 μm.

43. Avoid charging of the sections by attaching 1–2 mm carbon adhesive strips along the sides of the CNT tape, connecting it to the carbon conductive tape and the silicon.

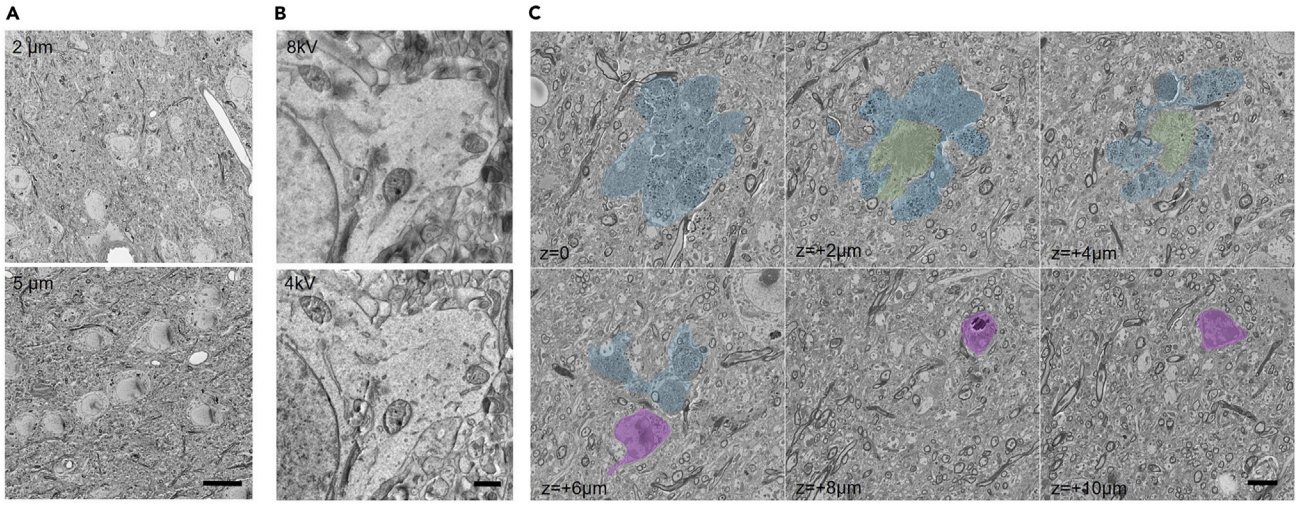

Figure 7. Scanning electron microscopy

(A) BSD images of mouse cortical tissue sectioned at 2 µm (top) and 5 µm (bottom) are shown. Scale bar, 5 µm.

(B) For semi-thick sectioning, a landing energy of 4 keV improves the resolution (bottom) compared to 8 keV (top). Scale bar, 1 µm.

(C) Cortex tissue of a familial Alzheimer's disease mouse was partitioned into 2 µm thick sections. Consecutive sections are shown highlighting the dystrophic neurites (blue), the amyloid core (green) and surrounding microglia (magenta). Scale bar, 5 µm.

⚠ CRITICAL: As semi-thick sections are more prone to charging compared to ultrathin sections, proper grounding is critical.

44. Store the wafer under vacuum or in a dry container until imaging.

Pause Point: months

45. Load the wafer into a SEM (Crossbeam Gemini 340, Zeiss)
46. Acquire serial section images using a backscatter detector (BSD) at 4 keV (high gain) at 7–8 mm WD and 30 or 60 µm aperture, 300 µA (Figure 7).

Note: For semi-thick sections, the resolution gets worse with 4–8 keV due to higher penetration depth of the electron beam and consequently more signal originating from deeper regions.

47. Generate a wafer overview map at 1,000 × 1,000 to 3,000 × 3,000 nm in ATLAS 5 Array Tomography (Fibics).
48. Map and image sections at medium resolution (60 × 60 to 100 × 100 nm).
49. Acquire regions of interest from these section sets at 10 × 10 nm (2 µs dwell time, line average 2) (Figure 7).

Note: Longer dwell times and/or higher resolution usually results in charging.

Pause Point: months

Target identification and preparation

Timing: 1 h to 2 days

In this step of the protocol, we describe how to find target regions based on light and/or electron microscopy data.

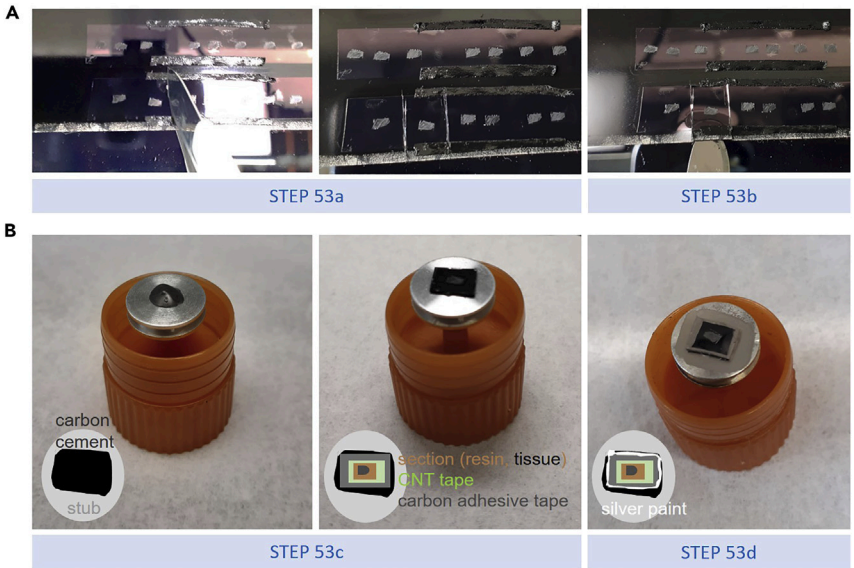

Figure 8. Remounting a section of interest onto a FIB stub

(A) Photos showing the detachment of a section of interest from the silicon wafer. The section including the carbon adhesive tape is excised and lifted using a scalpel.

(B) The section of interest is mounted on a SEM stub (middle) using carbon cement (left) and silver paint (right) to optimize conductivity. Adapted figure reprinted with permission from Figure S3, Kislinger et al. (2020).

50. Align images in TrakEM2 using a combination of automated and manual steps, registered and analyzed in Fiji (Schindelin et al., 2012).
51. Depending on the scientific question, target recognition requires either the 3D morphology of an object or specific ultrastructural features on any of the sections. Vasculature, cell somata or other structures in the range of 2–10 μm can be segmented and reconstructed using VAST (Berger et al., 2018).
52. The section(s) with the selected structure of interest have to be identified in order to subject it to FIB-SEM analysis.
53. Transfer the section of interest onto a FIB stub (Figure 8).
    a. Use a scalpel to cut the carbon adhesive around the section of interest on the two sides adjacent to the space between the previous and next section.
    b. Lift the adhesive tape with the section of interest with the scalpel.
    c. Add carbon cement (Science Services) on a FIB stub and position the sample onto it. Remove remaining carbon cement and let dry for 2 h.
    d. Use silver paint to surround the section helping to ground the sample. Take care not to touch the section at any point. Let the prepared sample dry.
    e. Coat with a thin carbon layer using a sputter coater (e.g., Quorum). If the coating is too thick, relocation of the area of interest is impaired. (recommended settings: 2 pulses at 50 A for 5 s each, 10 s interval)
    f. Store the sample under vacuum until the imaging session.

Pause Point: months

FIB-SEM acquisition of target region

Timing: 2–5 days

After identification of the region of interest, FIB-SEM is applied to generate a high resolution dataset at isotropic voxels (Figures 9A–9C). Target structures are segmented and rendered (Figures 9D and 9E).

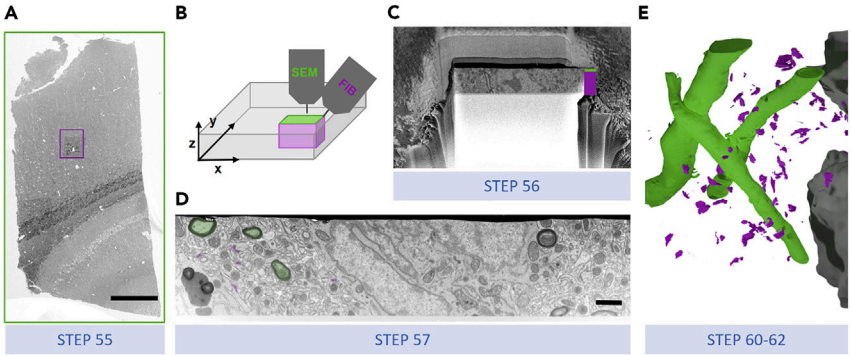

Figure 9. Targeted FIB-SEM in mouse cortex

(A) The BSD image (green) of a mouse cortex sample is shown including the target area (magenta) for FIB-SEM investigation. Scale bar, 200 μm.

(B) Schematics of targeted FIB-SEM on a semi-thick section. The SEM surface scanning area (green) to identify the target region volume (magenta) are sketched and correspond to the SEM image in (C).

(C) The milled trench is exposing the tissue cross-section (5 μm). The region of interest is covered by carbon (black) and platinum (light gray).

(D) Single InlenseDuo image of a semi-thick cross. Myelinated axons and postsynaptic densities that are subjected to segmentation are highlighted (green, magenta, respectively). Scale bar, 1 μm. Dataset from Kislinger et al. (2020)).

(E) Segmentation and rendering of myelinated axons (green9, PSDs (magenta) and nuclei (gray). Adapted with permission from Figure 4E, Kislinger et al. (2020).

54. Load the sample into the SEM (e.g., Crossbeam 340, Zeiss) and image the section using the SE detector at 5–8 kV landing energy and 1.5–2 mm working distance.
55. The previously acquired SEM or LM image yielding the region of interest can be imported directly into ATLAS 5 or, alternatively, displayed in any suitable software. Identify the region of interest according to the rough position within the section and suitable landmarks (e.g., surrounding cell bodies, blood vessels etc.).
56. Preparation for FIB-SEM using ATLAS 5 3D.
    a. Find the eucentric point.
    b. Find the coincidence point.
    c. Deposit a carbon layer at the site of interest.
    d. Mill a trench with x and y dimensions covering the area of interest and a depth exceeding the sample thickness by 3–5 μm (e.g., 8–10 μm for 5 μm thick sections).
    e. Polish the cross-section.
    f. Set up the FIB-SEM run choosing suitable imaging and milling conditions (InlenseDuo and SE detectors, dwell time is 2–3 μs, line average 2–3, resolution 5 × 5 × 5 to 10 × 10 × 10 nm). Typically, an imaging region covering the entire cross-section is chosen.
    g. Chose a region for the automated focus acquisition every 30 min.
57. Acquire the entire region if interest (typically 10–50 × 10–50 × 6 μm for a 5 μm thick section).
    a. Start with larger section thicknesses (typically 20–50 nm) and readjust to the target z resolution close to the region of interest.
    b. Readjust the window for the region of interest
    c. Monitor the FIB-SEM run and adjust parameters if needed.

Alternative: The FIB-SEM run can be set up in SmartFIB or any comparable standard FIB software.

Note: The FIB-SEM procedure is shortly summarized here, as there is no special aspect to be considered compared to standard tissue samples except the small trench depth. For complete details see your FIB-SEM microscopy handbook or refer to the literature (Knott et al., 2008, Xu and Hess, 2011, Xu et al., 2017).

Image alignment, segmentation, and rendering

Timing: 2 days to 3 weeks

This section lists image analysis steps for FIB-SEM data.

58. Align FIB-SEM image stacks in Fiji TrakEM2 (Schindelin et al., 2012, Cardona et al., 2012).
59. Combine InlenseDuo and SE detector images in the desired ratio.
60. Objects of interest can be segmented using VAST (Berger et al., 2018).
61. Segmented objects can be exported as .obj files.
62. Obj. files are rendered by Blender (Community, 2017) in order to obtain a plastic 3D model and to generate movies.

Note: The outlined image analysis part of the protocol is identical to standard volume EM image analysis.

Pause Point: infinite

EXPECTED OUTCOMES

The presented workflow describes how to generate 3D ultrastructural data of a target area within a larger tissue sample. Resin procuring and heated microtomy facilitate the generation of 20–100 serial sections in the range of 500 nm to 10 μm thickness. Sections are transferred onto plastic tape and mounted on silicon wafers. Tissue partitioning into a screenable library enables the search for regions of interest (up to 50 × 50 × 10 μm) at the ultrastructural level with adaptable resolution and re-inspecting options. If larger fiducial objects like the vasculature are sufficient for relocation, the transparent support tape allows for an even quicker searching procedure either solely by transmitted light microscopy or as a step before imaging by SEM. After identifying sections with areas of interest, they are remounted for FIB-SEM examination. FIB preparation exposes the cross-section of the selected semi-thick section and FIB-SEM perpendicular to the surface generates an isotropic volume of the area of interest with nanoscale resolution (5 × 5 × 5 nm).

LIMITATIONS

The microtomy step is prone to artifacts like surface heterogeneities or cracks. Heavily contrasted and heterogeneously stained regions are mostly susceptible to sectioning problems. So far, we have worked with diamond knives for a maximum of 2,000 semi-thick sections and sharpening could be a financial bottleneck and time-consuming. Staining, embedding and sectioning conditions have to be adapted to the given tissue sample and scientific question similar to existing volume EM techniques (Baena et al., 2019). The ATUM-FIB approach has so far been tested for up to 100 serial 5 μm thick sections but has to be optimized for large-scale investigations (Xu et al., 2017, Hayworth et al., 2015), especially for sections thicker than 5 μm. For neurite tracing intersection tissue loss has to be considered more carefully. If the biological object of interest is contained within more than two consecutive sections image stitching becomes a greater challenge. Both, FIB milling direction and image stack alignment in three dimensions need become critical (Hayworth et al., 2015).

TROUBLESHOOTING

Problem 1

During sectioning water condensates on the sample block (steps 28 and 29).

Potential solution 1

Cool down the knife or increase the sample temperature by approaching the infrared lights.

Problem 2

Sections are not taken up by the tape (step 29).

Potential solution 2

Decrease the sectioning and tape speed and use a brush to guide the sections onto the tape. Alternatively, expand the sectioning window to avoid complete stopping of the collection process.

Problem 3

Surface heterogeneities and cracks (as observed during step 28, 35b, or 46)

Potential solutions 3

- Polymer contaminations are assembling more quickly during semi-thick compared to ultrathin sectioning on the diamond. Knife cleaning using a styrofoam stick is recommended every 500 sections. Consider knife sharpening.
- Vary either knife or sample temperature.
- Vary the precuring time (10–20 h).
- Decrease the block face area and/or block face shape or vary the ratio of tissue to empty resin. Squared or rectangular shaped block faces are preferred, but a diamond shape can be beneficial as the cutting edge is reduced. However, diamond sections tend to rotate in the water bath.
- Vary the rOTO protocol. Heavily contrasted tissue is prone to disintegrate from the resin. Reduce the osmium (1%) or uranyl acetate (0.5%) concentrations or perform variations of the Tapia rOTO, Hua rOTO or fBROPA protocols (Tapia et al., 2012, Hua et al., 2015, Genoud et al., 2018).
- Change the resin formulation.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Martina Schifferer (martina.schifferer@dzne.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The datasets supporting the current study have not been deposited in a public repository but are available from the corresponding author on request.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100232.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.M. and M. Schifferer; methodology, H.G., G.K., and M. Schifferer; investigation, G.K. and M. Schifferer; resources, H.G.; visualization, G.K. and M. Schifferer; supervision,

funding acquisition, and writing – review & editing, M.K., M. Simons, and T.M.; writing – original draft and rroject administration, M. Schifferer.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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