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.
Protocol

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

Georg Kislinger,1,2,3 Helmut Gnägi,4 Martin Kerschensteiner,2,5,6 Mikael Simons,1,2,3 Thomas Misgeld,1,2,3 and Martina Schifferer1,2,7,8,*

1German Center for Neurodegenerative Diseases (DZNE), Munich 81377, Germany
2Munich Cluster of Systems Neurology (SyNergy), Munich 81377, Germany
3Institute of Neuronal Cell Biology, Technical University Munich, Munich 80802, Germany
4Diatome SA, Helmstrasse 1, 2560 Nidau, Switzerland
5Institute of Clinical Neuroimmunology, University Hospital, Ludwig-Maximilians-University Munich, Munich 81377, Germany
6Biomedical Center (BMC), Faculty of Medicine, Ludwig-Maximilians-University Munich, Planegg-Martinsried 82152, Germany
7Technical contact
8Lead contact
*Correspondence: martina.schifferer@dzne.de
https://doi.org/10.1016/j.xpro.2020.100232

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.
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# P7719-25 |
| 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 |
| Panoramic 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# WSM40525200P13345NN1 |
| Standard oven (e.g., Incu-line IL 10) | WWR | Cat# 390-0384P |
| Chemicals, peptides, and recombinant proteins |        |            |

(Continued on next page)
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.
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 |
|---------------------------------------|---------------------|--------|
| 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: 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.

| 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 |
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**).
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.

### 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 x 10 min | off | 250 W | 3 x 10 min |
| 2% osmium tetroxide, 2.5% potassium hexacyanoferrate in 0.1 M cacodylate buffer | 20°C–24°C | 6 x 1 min | on | 300 W | 2 h |
|                                  | 20°C–24°C   | 1 h (shaker) | – | – | – |
| 0.1 M cacodylate buffer          | 20°C–24°C   | 2 x 10 min | off | 250 W | 2 x 10 min |
| Water                            | 20°C–24°C   | 1 min | on | 250 W | 10 min |
| 1% TCH                           | 4°C–24°C    | 25 min | on | 200 W | 45 min |
| Water                            | 20°C–24°C   | 3 x 10 min | on | – | 3 x 10 min |
| 2% osmium tetroxide in water     | 20°C–24°C   | 6 x 1 min | on | 300 W | 2 h |
|                                  | 20°C–24°C | 1 h (shaker) | – | – | – |
| Water                            | 20°C–24°C   | 3 x 10 min | on | – | 3 x 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 |
| 100% LX112                       | 20°C–24°C   | 1 h | – | – | – |
| 100% LX112                       | 20°C–24°C   | 10 min | on | 250 W | 15 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.
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.
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 months. 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.
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 \( \mu \)m \( \times \) 5) using the 20\( \times \) 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\( \times \) or the 40\( \times \) 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.

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**Pause Point:** months
Serial scanning electron microscopy

Timings: 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 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.
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.
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).
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 \( \mu \text{m} \) (e.g., 8–10 \( \mu \text{m} \) for 5 \( \mu \text{m} \) 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 \( \mu \text{s} \), line average 2–3, resolution 5 \times 5 \times 5 \text{ to } 10 \times 10 \times 10 \text{ 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 \times 10–50 \times 6 \( \mu \text{m} \) for a 5 \( \mu \text{m} \) thick section).
   a. Start with larger section thicknesses (typically 20–50 \text{ 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 tBROPA 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.

ACKNOWLEDGMENTS
This work was supported by DFG under Germany’s Excellence Strategy within the framework of the Munich Cluster for Systems Neurology (EXC 2145 SyNergy, ID 390857198) and the TRR 274/1 2020, 408885537 (project Z01). T.M.’s lab was also supported by DFG FOR2879, A03 and SFB/TRR274, projects B03 and C02. We thank Katalin Völgyi and Ozgun Gokce for providing fixed brain samples, Richard Schalek, Mark Terasaki, and Gerhard Wanner for valuable scientific and technical advice, and Felix Beyer and Birgit Kunkel for technical assistance.

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 project administration, M. Schifferer.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES

Baena, V., Schalek, R.L., Lichtman, J.W., and Terasaki, M. (2019). Serial-section electron microscopy using automated tape-collecting ultramicrotome (ATUM). Methods Cell Biol. 152, 41–67.

Berger, D.R., Seung, H.S., and Lichtman, J.W. (2018). VAST (Volume Annotation and Segmentation Tool): efficient manual and semi-automatic labeling of large 3D image stacks. Front. Neural Circuits 12, 88.

Cardona, A., Saalfeld, S., Schindelin, J., Arganda-Carreras, I., Preibisch, S., Longair, M., Tomancak, P., Hartenstein, V., and Douglas, R.J. (2012). TrakEM2 software for neural circuit reconstruction. PLoS One 7, e38011.

Community, B.O. (2017). Blender—a 3D modelling and rendering package (Blender Foundation, Blender Institute Amsterdam).

Genoud, C., Titze, B., Graff-Meyer, A., and Friedrich, R.W. (2018). Fast homogeneous en bloc staining of large tissue samples for volume electron microscopy. Front. Neuroanat. 12, 76.

Hayworth, K.J., Xu, C.S., Lu, Z., Knott, G.W., Fetter, R.D., Tapia, J.C., Lichtman, J.W., and Hess, H.F. (2015). Ultrastructurally smooth thick partitioning and volume stitching for large-scale connectomics. Nat. Methods 12, 319.

Hua, Y., Laserstein, P., and Helmstaedter, M. (2015). Large-volume en-bloc staining for electron microscopy-based connectomics. Nat. Commun. 6, 7923.

Kislinger, G., Gnägi, H., Kerschensteinert, M., Simons, M., Misgeld, T., and Schifferer, M. (2020). Multiscale ATUM-FIB microscopy enables targeted ultrastructural analysis at isotropic resolution. iScience 23, 101290.

Knott, G., Marchman, H., Wall, D., and Lich, B. (2008). Serial section scanning electron microscopy of adult brain tissue using focused ion beam milling. J. Neurosci. 28, 2959–2964.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682.

Tapia, J.C., Kasthuri, N., Hayworth, K.J., Schalek, R., Lichtman, J.W., Smith, S.J., and Buchanan, J. (2012). High-contrast en bloc staining of neuronal tissue for field emission scanning electron microscopy. Nat. Protoc. 7, 193–206.

Xu, C., and Hess, H. (2011). A closer look at the brain in 3D using FIB-SEM. Microsc. Microanal. 17 (S2), 664–665.

Xu, C.S., Hayworth, K.J., Lu, Z., Grob, P., Hassan, A.M., Garcia-Cerdan, J.G., Nyogi, K.K., Nogales, E., Weinberg, R.J., and Hess, H.F. (2017). Enhanced FIB-SEM systems for large-volume 3D imaging. eLife 6, e25916.