Video Article

Multimodal Hierarchical Imaging of Serial Sections for Finding Specific Cellular Targets within Large Volumes

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Abstract

Targeting specific cells at ultrastructural resolution within a mixed cell population or a tissue can be achieved by hierarchical imaging using a combination of light and electron microscopy. Samples embedded in resin are sectioned into arrays consisting of ribbons of hundreds of ultrathin sections and deposited on pieces of silicon wafer or conductively coated coverslips. Arrays are imaged at low resolution using a digital consumer like smartphone camera or light microscope (LM) for a rapid large area overview, or a wide field fluorescence microscope (fluorescence light microscopy (FLM)) after labeling with fluorophores. After post-staining with heavy metals, arrays are imaged in a scanning electron microscope (SEM). Selection of targets is possible from 3D reconstructions generated by FLM or from 3D reconstructions made from the SEM image stacks at intermediate resolution if no fluorescent markers are available. For ultrastructural analysis, selected targets are finally recorded in the SEM at high-resolution (a few nanometer image pixels). A ribbon-handling tool that can be retrofitted to any ultramicrotome is demonstrated. It helps with array production and substrate removal from the sectioning knife boat. A software platform that allows automated imaging of arrays in the SEM is discussed. Compared to other methods generating large volume EM data, such as serial block-face SEM (SBF-SEM) or focused ion beam SEM (FIB-SEM), this approach has two major advantages: (1) The resin-embedded sample is conserved, albeit in a sliced-up version. It can be stained in different ways and imaged with different resolutions. (2) As the sections can be post-stained, it is not necessary to use samples strongly block-stained with heavy metals to introduce contrast for SEM imaging or render the tissue blocks conductive. This makes the method applicable to a wide variety of materials and biological questions. Particularly prefixed materials *e.g.*, from biopsy banks and pathology labs, can directly be embedded and reconstructed in 3D.

Video Link

The video component of this article can be found at https://www.jove.com/video/57059/

Introduction

For reconstructing large volumes of tissue at ultrastructural resolution a number of different imaging approaches based on SEM have been used[1]: Comprehensive reviews are available *e.g.*, for SBF-SEM[2], FIB-SEM[3], and Array Tomography (AT)[4]. While for the latter method the sample material is preserved as an array of serial sections on a substrate, SBF-SEM and FIB-SEM are destructive methods, working on the sample block and consuming it during imaging. Due to the charging of the resin in the SEM, they also depend on strongly metalized sample blocks[5].

On the other hand, identifying certain cells or structures of interest within a tissue sample can profit particularly from correlative light and electron microscopy (CLEM)[6,7,8]. Using FLM for targeting precludes the application of large amounts of heavy metal since this would quench the fluorescence signal[9]. For such only slightly metalized samples, AT is the method of choice since arrays may easily be post-stained with heavy metal after LM imaging. Moreover, almost any sample type may be used for AT, even routine samples from the pathologist's treasure chest[10].

Another big advantage of AT is the potential for hierarchical[11] or multi-resolution imaging[12]: It is not necessary to image everything at high-resolution, as targets may be selected in a different modality (*e.g.*, FLM) or in low-resolution SEM images. Imaging only the interesting regions of a tissue or cell population at high-resolution saves digital data storage space and produces smaller image data sets, which are easier to

handle. Here, the AT workflow is demonstrated using a rather weakly metalized sample: high pressure frozen plant roots (*Arabidopsis thaliana*) embedded in hydrophilic resin.

How the arrays are prepared, stained, and imaged in both FLM and SEM, and how the image stacks are registered are explained. Also, how the 3D reconstruction of the FLM volume can be used to select specific cells for imaging in the SEM at nanoscale resolution is illustrated.

Protocol

NOTE: Sample blocks should be polymerized and contain some heavy metal. Fixation and embedding protocols for the two samples shown in **Figure 1A-B** have been described elsewhere[11]. In short, the sample shown in **Figure 1A** was chemically fixed, stained *en bloc* first with 1% OsO_4, then with 1% uranyl acetate, and embedded in Spurr's resin. The sample shown in **Figure 1B** was high pressure frozen, freeze substituted with 0.4% uranyl acetate in acetone, and embedded in Lowicryl HM20 resin. Use powder free gloves for the next preparation steps.

1. Creating Arrays

1. **Trimming of the sample block**
   NOTE: Always tighten screws well when inserting parts.
   1. Insert the sample block into the sample holder of the ultramicrotome, place the holder into the trimming block, and slide it into the lower stage of the ultramicrotome.
   2. Trim away resin around the embedded tissue with a razor blade leaving only a small rim of resin around the sample. Trim from the top until the target is reached.
      NOTE: The shape of the block-face may be trapezoid or rectangular (we have successfully used both shapes). It is important to use the longer sides of the rectangle as the leading and trailing edge to ensure that a large area is covered by the glue mixture stabilizing the ribbons.
   3. Insert the sample holder into the arm of the ultramicrotome and replace the trimming block in the lower stage by the knife holder. Insert a diamond trim knife (normally 45°), into the knife holder. Align the block-face exactly parallel to the trim knife edge.
      NOTE: To produce exactly parallel leading (bottom) and trailing (top) edges, turn or move only the knife to trim opposite sides. For large volumes (more than several hundred sections), a 90° trim knife is advantageous.
   4. Smooth all four sides, then rotate the sample holder so that the leading and trailing edge are now in the horizontal position.
   5. Carefully coat the leading and trailing sides of the block with adhesive mixture. Use a tiny brush formed from a few hairs fixed to a toothpick[13]. Perform this step quickly because the solvent of this mixture evaporates within seconds. Do not contaminate the block-face with this mixture. Let the coated sample block dry for 5-10 min.
      NOTE: For larger numbers of sections (>200), it may be better to coat the leading edge only, because with time, a bulge of glue might build up at the trailing edge, and potentially pull back sections over the knife-edge.

2. **Preparation of the substrate**
   1. Cut pieces of silicon wafers to a size that fits into the knife boat (roughly 2 x 2.5 cm^2 for the Jumbo knife). If needed, mark the wafer pieces (numbers or letters) with a diamond scriber before cleaning, or with permanent marker after cleaning. Clean the silicon wafer manually with isopropanol and lint-free tissue.
      NOTE: For correlative imaging use indium-tin-oxide (ITO)-coated glass coverslips. They must be handled very carefully, and extra cleaning is not necessary.
   2. Fix the substrate to one end of the carrier plate using a removable adhesive.
      NOTE: Alternatively, the substrate edges may be pinned down parallel to the knife-edge using two stripes of adhesive tape.
   3. Plasma activate (glow discharge) the substrate with air to obtain a hydrophilic surface. This should be done in such a way that a drop of water placed onto the substrate spreads to a very thin film (low contact angle, **Figure 2C, D**). Hydrophilization parameters depend on the plasma device used; for the parameters used here, see **Table of Materials**.
      NOTE: Plasma activation is very volatile, so perform this immediately before substrate usage.
   4. Insert the carrier into the clamp of a substrate holder, with the mounted substrate closer to the knife-edge to achieve optimal wetting of the substrate in the knife boat.
      NOTE: A detailed description of the substrate holder, including the computer-aided design (CAD) drawings, is given in Wacker *et al*.[11]

3. **Sectioning Preparation**
   1. Insert a Jumbo diamond knife into the knife holder, set the clearance angle (0° for Jumbo knife), and fill the knife boat with distilled water. Approach the knife to a distance of 1–2 mm to the sample.
   2. Lower the substrate into the water using screws 1–3 (**Figure 2A**) of the substrate holder. Check that the waterline is located in the upper third of the substrate.
      NOTE: Check that the glue between the substrate and carrier plate is well cured by nudging the substrate with clean forceps. It should not move.
   3. Because it is hard to see the ground clearance when using a silicon wafer, lower the substrate until you feel it touch the floor. Now raise the substrate a small amount. Make sure that neither the substrate nor the carrier touches the knife boat while cutting.
   4. Use a syringe or pipette to adjust the water level in the boat. While watching through the binocular, add or remove water until the full area of the water surface shows a homogenous reflection of the top light illumination of the ultramicrotome.
   5. Switch on the bottom light of the ultramicrotome. Ensure that the arm is in the middle position and not in retraction using the hand wheel of the ultramicrotome. Approach the knife to the sample until the reflection of the knife-edge is visible on the block face.
   6. Use the adjustment options of the ultramicrotome to align the sample to the knife. First rotate the knife, then rotate and tilt the sample.
      NOTE: It is properly aligned if the light stripe, which can be seen in the gap between the sample and the knife, shows parallel edges (straight parallel lines and not wedge shaped).

7. Check the inclination of the sample: Make sure that the light stripe does not become thicker or thinner while moving the sample up and down. If needed, use the arc adjustment screw to correct this. Move the knife closer to the sample until it is just above the block-face (but does not touch it).
8. Set section thickness (feed), cutting speed, and cutting window at the control unit.
9. Start sectioning. If necessary, wait until the first full section is being cut. Cut some sections to be sure that they stick together to form ribbons (otherwise the glue has to be applied again). Start with a high feed value (maximum, 200 nm for the Ultra knife) until the first full section is being cut. Then, set the desired feed value. For adequate ribbon stability, a section thickness of 100 nm and a cutting speed of 1 mm/s is a good starting point. The lowest achievable section thickness is around 60 nm, depending on sample quality.
10. Stop sectioning. Remove all unneeded (partially cut) sections from the knife-edge and boat using an eyelash/cat's hair. If there is a lot of small debris floating around, remove the water completely with a pipette and fill the boat with fresh water. Now the process is ready for the first productive ribbon.

4. **Sectioning**
   1. Start sectioning. Once a number of sections (the actual number depends on the size of sections and substrate) has been cut, stop the sectioning process and release the ribbon from the knife-edge by gently stroking over the knife-edge with an eyelash[14] or better yet, with a very soft hair from a cat's fur.
   2. Manipulate (push/pull) the ribbon with the eyelash towards the substrate and attach the first section to the substrate.
      NOTE: It is necessary to gently push the ribbon until it sticks to the dry part of the substrate.
   3. Continue sectioning and attaching the ribbons to the substrate. Start on one side and move gradually over to the other with each new ribbon.
      NOTE: Avoid massive water movements to avoid loosening the already attached ribbons. The same is true for air currents. Use the breath shield delivered with the ultramicrotome. In unfavorable environmental conditions, an enclosure for the ultramicrotome is recommended[15].
   4. When the substrate is completely covered with ribbons (usually 4–5 ribbons are feasible), gently lift-out the substrate from the knife boat using the micromanipulator screws of the substrate holder.
      NOTE: Suitable movements are: lift up vertically (screw 1) and rotating/tilting out (screw 3), or a combination of both.
   5. Let the ribbon array dry before storing it in a dust-free environment. After drying, remove the adhesive mounted substrate as soon as possible from the carrier (on the same day, otherwise the substrate might be too hard to remove or may even break during demounting).

2. Staining for the LM Imaging

NOTE: Different staining/labeling methods are possible, including immunofluorescence protocols. Here a direct, rather unspecific stain is chosen to outline the cell walls.

1. **Propidium iodide staining**
   1. Cover the bottom of a large glass Petri dish (30 cm diameter) with parafilm and line the edge of the dish with wet tissue to build a humid chamber.
   2. Use approximately 300-500 µL of solution per coverslip. Place one drop for each coverslip on the parafilm and put the glass upside down onto the drop, so that the sections are in contact with the staining fluid. Cover the dish and wrap it with aluminum foil to protect the samples from light. Incubate the samples for 16 h at 4 °C.
   3. Remove the coverslip with forceps and wash it by moving it up and down in a 100-mL beaker filled with 80 mL of distilled water. Repeat this step in another beaker with fresh water. Dry the coverslip carefully with compressed air.

3. Recording the Image Stack in the FLM

1. Place the coverslip on the stage of a common wide-field FLM.
2. Choose the appropriate filter set (**Table of Materials**) for the fluorescence to be observed.
3. With a suitable objective lens, take an image of the object in each section: try to fill the field of view and keep the orientation constant. For the root tip, a 40X air objective was used.
   NOTE: If the ribbons are not perfectly straight, a rotating stage can help to reorient the sections while taking images. If possible, center the image on a specific feature or keep a feature such as the edge of the section at an equal distance to the edge of the recorded image.
4. If possible, use 16 bit to limit saturated pixels and keep the exposure time constant.

4. Registration of the FLM Image Stack

1. Import the image series into Fiji[16] as a virtual stack.
2. Open a new TrakEM[17] (blank) from the file menu.
3. Right click into the image field, and import the stack into TrakEM as "One slice per layer".
4. Align layers (right click into image field), set the range (first image to last), and choose none as the reference. For all settings, use the default values, and choose rigid as the desired transformation.
5. When the registration is finished and satisfactory, save the aligned dataset by right click and choose export. Make flat image, set the range from first to last image, and let the software show the resulting stack. Save the stack in tif-format.

5. Staining and Mounting for the SEM Imaging

NOTE: For preparing staining solutions see **Table of Materials**. Solutions may be stored at 4 °C for up to 12 months, protected from light and air.

Caution: Lead citrate and uranyl acetate contain heavy metals which are toxic. Wear gloves and dispose the waste according to the local authorities' instructions.

1. Cover the bottom of a large glass Petri dish (30 cm diameter) with parafilm and line the edge of the dish with wet tissue to build a humid chamber.
   NOTE: It is important that several pellets of NaOH are positioned within the Petri dish near the staining drops to prevent excessive precipitation of lead citrate.
2. **Uranyl acetate staining**
   1. Centrifuge the uranyl acetate solution at 2,680 x g for a few seconds to sediment small particles.
   2. Use approximately 300–500 µL of solution per coverslip. Place one drop for each coverslip on the parafilm and put the glass upside down onto the drop, so that the sections are in contact with the staining fluid.
   3. Incubate for 10 min at room temperature, and cover the dish during staining.
   4. Remove the coverslip with forceps and wash by moving it up and down in a beaker filled with distilled water (see step 2.1.3).
3. **Lead citrate staining**
   1. During the uranyl acetate incubation, prepare the lead citrate solution.
      NOTE: Lead citrate should always be filtered immediately before use to remove any precipitates. Also centrifuge the lead citrate solution at 2,680 x g for a few seconds as in step 5.2.1.
   2. Use approximately 300–500 µL of solution per coverslip. Place one drop for each coverslip on the parafilm immediately before washing the coverslips after the uranyl acetate staining, and put the glass upside down onto the drop, so that the sections are in contact with the staining fluid. Place the drops (300–500 µL) on the parafilm immediately before washing the coverslips after the uranyl acetate staining.
      NOTE: To avoid formation of precipitates, do not breathe onto the lead citrate droplets.
   3. Place the washed coverslip upside down onto the drop (there is no need to dry it).
   4. Incubate for 5 min at room temperature, and cover the dish during the staining.
   5. Remove the coverslip with forceps and wash it as described above in a beaker with fresh water (step 5.2.4).
4. Dry the coverslip carefully with compressed air.
5. **Mounting samples for the SEM imaging**
   1. Mount the silicon wafers on aluminum stubs with a sticky carbon pad.
      NOTE: ITO-coated coverslips may either be mounted using silver paint and Cu-tape — make sure the conductive surface is connected to the stub — or with carbon pads as above. In that case, a conductive connection from the ITO-surface to the stub can be made with a drop of silver paint.

6. Hierarchical Imaging in the SEM

NOTE: In a field emission SEM, choose a low primary energy (3 kV or lower), a beam current in a range from 50 to 800 pA to avoid charging, and a suitable working distance for efficient collection of secondary and/or back-scattered electrons. Selection of the beam current depends on the sample properties (*e.g.*, embedding resin); the electron dose will also be a compromise between a small current (less harmful to the sample) and a high current, which is beneficial for imaging speed and therefore lowers the total image acquisition time. Dedicated detectors for back-scattered electrons provide good contrast, are less sensitive to charging of the sample, and show less of the sample's surface artifacts (folds, knife marks). Contrast and brightness should be adjusted such that the histogram is centered.

1. **SEM imaging**
   1. First define the four corners of the array by grabbing an image of each corner at low magnification, about 100x. Create a region of interest (ROI) enclosing the whole array. Assign an imaging protocol with the following parameters: Use a secondary electron (SE) detector, which allows for high speed imaging at a large image pixel size (for example 1,000 nm), and a short dwell time (e.g., 0.2 µs).
      NOTE: To overcome electron optical limitations at a large scanning fields of view (FOV), which might result in distortions at the periphery of the images, use dedicated low magnification modes (provided by most SEM manufacturers) or use medium, 1 to 2 k scan fields for single images.
   2. Generate a section set by creating a ROI outlining just the tissue in the first section. Clone it to all subsequent sections using the stamp tool. Rotate the ROIs when needed to accommodate bent ribbons.
   3. Record the image series using an intermediate pixel size (around 50 nm) and a dwell time long enough to identify and recognize the target structure. Use a FOV for single images in a range of 6-10 k pixels.
      NOTE: The Atlas 5 software can automatically collect mosaics composed of adjacent images to cover large ROI/section areas across the serial sections.
   4. Create a site set within this section set, containing the target structure for higher resolution SEM imaging. Make the ROI large enough to account for stage precision. Check and adjust the positions of the sites.
      NOTE: It is important to place the ROIs in such a way that the center, where the autofocus and autostigmation will be performed, does not sit on "empty" material with no structural detail, *e.g.*, vacuoles.
   5. Define the autofocus settings and check the performance over at least the length of a ribbon (*i.e.*, the longest distance that the stage has to travel) on a small ROI close to the site that will be imaged.
   6. Define an imaging protocol for the high-resolution SEM acquisition. To see membrane compartments, choose 3–5 nm image pixel size. Select a dwell time depending on the detector so that the image is not too noisy.
   7. Before starting the acquisition, define the focus values on at least the first section of each ribbon using the check protocol option.

8. Start the automated SEM imaging over the whole series of target ROIs.
9. Export the acquired data as image series, preferably in tif-format.

7. Registration of the SEM Image Stack

1. Import image series into Fiji as a virtual stack.
    NOTE: These will be large data files in the range of a few GB depending on the number of sections and the size of the ROI.
2. Crop the SEM image stack for further processing to an area as close to the structure of interest as possible, and adjust the brightness and contrast.
3. Open a new TrakEM[17] (blank) from the file menu.
4. Right click into the image field, and import the stack into TrakEM as "One slice per layer".
5. Align the layers (right click into the image field), choose least squares as mode, set the range (first image to last), and choose none as the reference. For the settings, use the default values and choose rigid as the desired transformation.
6. When the registration is completed and satisfactory, save the aligned dataset by right click and choose export. Make a flat image, set the range from first to last image, and let the software show the resulting stack. Save the stack in tif-format.

Representative Results

The workflow described here (**Figure 1**) starts with a sample embedded in a resin block. During the sample preparation, some heavy metal should be introduced into the tissue, but it is not necessary to use protocols optimized for rather strong metallization. **Figure 1A** shows a plant root (cress) block-stained conventionally with 1% OsO_4 and 1% uranyl acetate, while the *Arabidopsis* root in **Figure 1B** is only weakly metalized using 0.5% uranyl acetate. The latter sample type is best suited for correlative approaches as some heavy metals tend to quench fluorescence. With a dedicated substrate holder (**Figure 2**), arrays of several hundred sections can be produced (**Figure 1C**). After fluorescent labeling, such arrays are imaged in a standard wide-field FLM (**Figure 1D**), then stained with heavy metal solutions and imaged in a SEM at different resolutions (**Figure 1E-G**).

Important tools for the reproducible generation of arrays, particularly when placing several ribbons from the microtome's knife boat onto a substrate, are the substrate holder (**Figure 2A**, custom-designed in the authors' laboratory) and a Jumbo diamond knife with a boat large enough to accommodate microscope slides (**Figure 2B**). A flat meniscus, allowing good observation of the ribbons, is necessary and can be achieved by plasma cleaning of the substrate: A small droplet of distilled water should not form a lens-like structure on the substrate as in **Figure 2C** (untreated substrate), but a thin film (**Figure 2D**, plasma activated substrate). Under these conditions, ribbons attached to the dry part of an ITO-coated coverslip are easily visualized (**Figure 2E**) and can be observed and controlled during the lift-out of the substrate from the water.

As an example, arrays stained with propidium iodide to label the plant cell walls were imaged with a standard wide field FLM (**Figure 3A**). Since the sections are only 100 nm thick, even over-staining as shown here introduces little blurring. After registration, the two cells completely enclosed in the reconstructed volume were selected from the image stack (**Figure 3B**) for high-resolution imaging in 3D (see also **Supplemental Movie S1**). Following additional staining with uranyl acetate and lead citrate, the arrays were imaged in the SEM. **Figure 3C** shows an overview, recorded with 60 nm image pixels; the dark square in the center of the image indicates the position where the autofocus functions were executed, and the additional dose led to slight contamination. Appropriate ROIs in those serial sections (slices 51 to 248 of 435 slices in total) containing the two target cells selected in the FLM stack were then recorded with a 5 nm image pixel size (**Figure 3D**; see also **Supplemental Movie S2**).

Automated hierarchical imaging of the arrays in the SEM described here was done with the software/hardware platform solution ZEISS Atlas 5. First, an overview of the whole array was created using the SE detector, with very large (1,000 nm) image pixels and very low dwell time (**Figure 4A**). An ROI outlining only the tissue was placed on the first section and propagated to all other sections of the array. This section set was then recorded with 60 nm image pixels using a longer dwell time (**Figure 4B**). Finally, a site set, containing the two target cells plus one "layer" of surrounding cells to account for stage inaccuracy, was set up with the following parameters: ESB (Energy Selective Backscatter) detector, 5 nm image pixels, very long (40 µs) dwell time (**Figure 4C**). Zooming in to such an image shows subcellular detail (**Figure 4D**) such as vacuoles (V), mitochondria (M), nucleus (N), and endoplasmic reticulum (arrows). See also the **Supplemental Movie S3** for zooming in from an overview of the whole array to the subcellular detail of one target cell.

The array shown here (200 sections) plus an additional one of 250 sections took about 8 h to produce, one night to stain for LM, and one day to record (manually) at the FLM. Post-staining takes about 1-2 h in total, depending on the number of individual arrays. For SEM recording, a few hours are required to set up the Atlas run, and automated recording was 3–4 h for the intermediate resolution (60 nm pixel size) section set (200 sections, 450 x 200 µm^2) and about 5 days for the high-resolution (5 nm pixel size) ROI containing the two target cells (200 sections, 55 x 30 µm^2). Note that due to the low metal content of the sample shown here, a very slow scanning speed had to be used to reach a good signal-to-noise detection, which implied (for the currently available detector) a dwell time of 40 µs for the high-resolution ROI.

There are several steps in the whole workflow prone to pitfalls: Ideally ribbons should be more or less straight and placed in the right order (**Figure 5A**). However, bent (**Figure 5B**), curved (**Figure 5D**), or even broken ribbons are often produced. This can result due to incorrect trimming (leading and trailing edges not exactly parallel), or non-uniformly applied adhesive, but also from an asymmetric or unevenly infiltrated sample. Particularly troublesome are samples containing both soft and very hard components. The latter components may be difficult to infiltrate such as the cell wall of the plant roots shown here (**Figure 5C**). In that case, folds (arrowheads) can easily be caused by variable compression and relaxation during sectioning. For automated imaging in the SEM, curved ribbons are not a great problem, since the ROIs can be rotated to accommodate the curvature of the ribbon.

Another critical step in the protocol is staining: Inadequate washing can lead to residues on the section (**Figure 5E, F**), and in the worst case, cover the most interesting area (circle on one of the two target cells in **Figure 5F**). Also, dust (**Figure 5D**, strongly light scattering particles) introduced into the knife boat, *e.g.*, with a dirty substrate carrier, can cause serious problems: In the FLM, dust can be highly fluorescent (cf. some slices in **Supplemental Movie S1**) to such an extent that some registration algorithms do not function. The "align" function in TrakEM[17] however, can handle such stacks as demonstrated in **Supplemental Movie S1**.
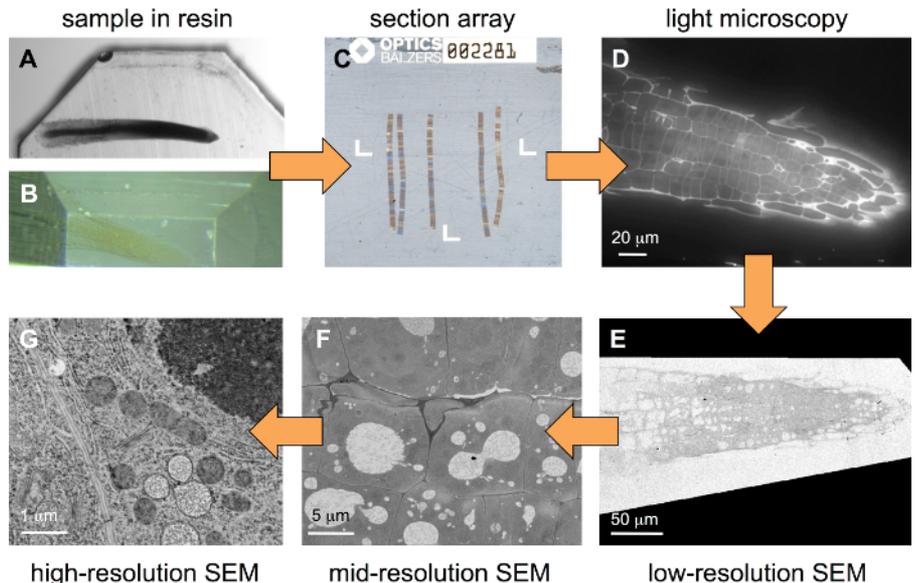
Figure 1: Workflow for the correlative hierarchical imaging. Starting from a sample embedded in a resin block (**A**, strongly metalized sample), the sample is first trimmed (**B**, weakly metalized sample) and then arrays consisting of several ribbons of serial sections (**C**), here placed on an ITO-coated coverslip, are produced using an ultramicrotome. After staining with a fluorescent dye, stacks of images are recorded in a wide-field FLM (**D**). After further staining rounds with heavy metal salts, stacks are imaged in a SEM (**E-G**) at different resolutions (image pixel sizes). Please click here to view a larger version of this figure.
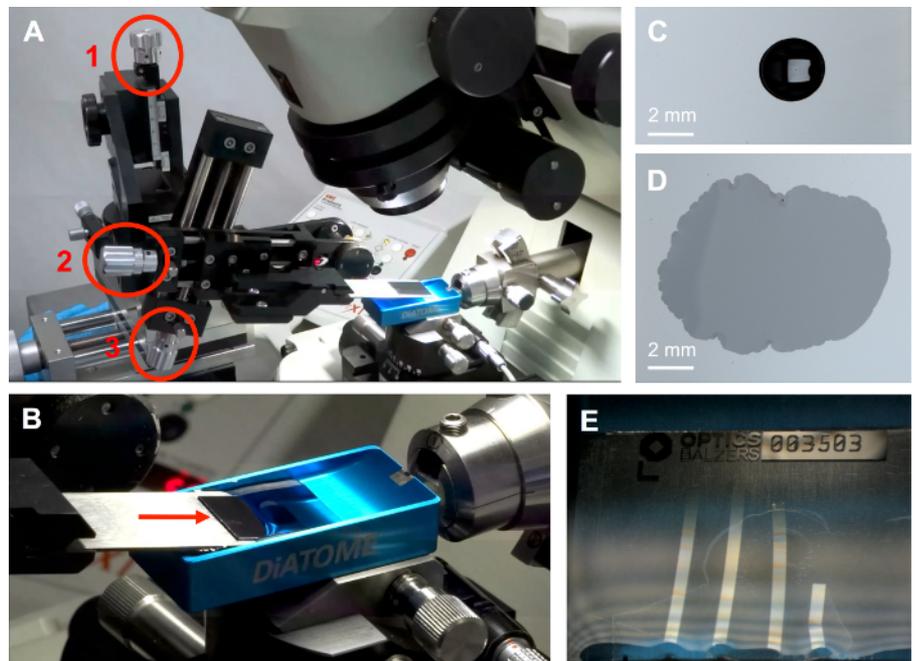

Figure 2: Tools for the preparation of arrays. Substrate holder assembled from micromanipulators with seven axes of movement attached to a standard ultramicrotome (**A**): the screws, highlighted with circles, are for vertical (1) and horizontal (2) movement and for tilting (3) of the substrate carrier. The jumbo diamond knife with an oversize boat to accommodate large substrates (arrow), here with a piece of plasma-activated silicon wafer mounted onto a slide-sized aluminum carrier (**B**). 20 µL drops of distilled water placed onto an untreated silicon wafer substrate (**C**) or on a plasma-activated substrate (**D**). Four ribbons floating in the knife boat, attached to an ITO-coated coverslip by their lower ends. Please click here to view a larger version of this figure.
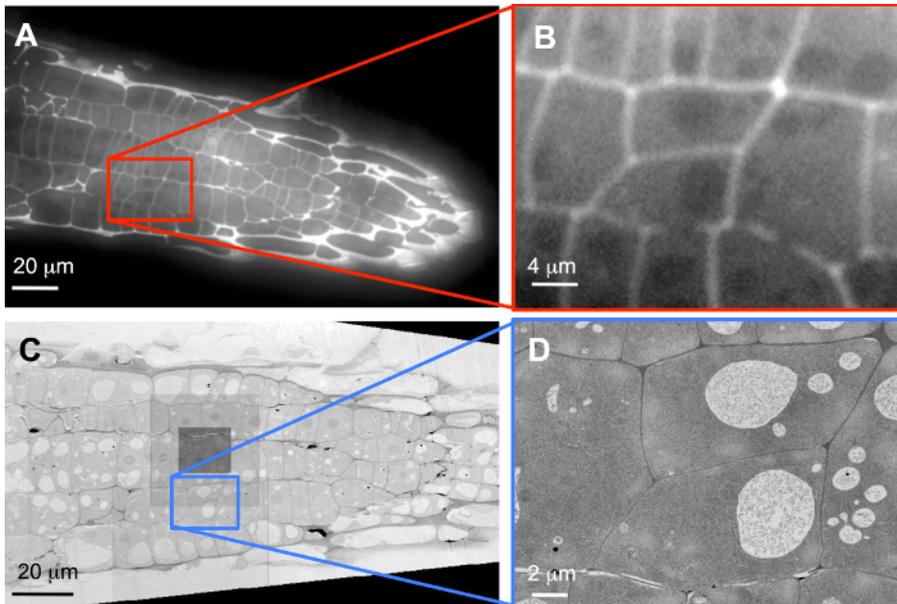
Figure 3: Correlation of the LM data with SEM data. Overviews (**A**, **C**) and target cells (**B**, **D**) recorded with FLM (**A**, **B**) and SEM (**C**, **D**). (**B**) is a software zoom, and the original data were recorded with a 40X objective lens on a 1,388 x 1,040 pixel camera chip, while (**C**) is recorded with 60 nm image pixel size, and (**D**) with 5 nm image pixel size, illustrating the true increase in resolution in the SEM. Please click here to view a larger version of this figure.
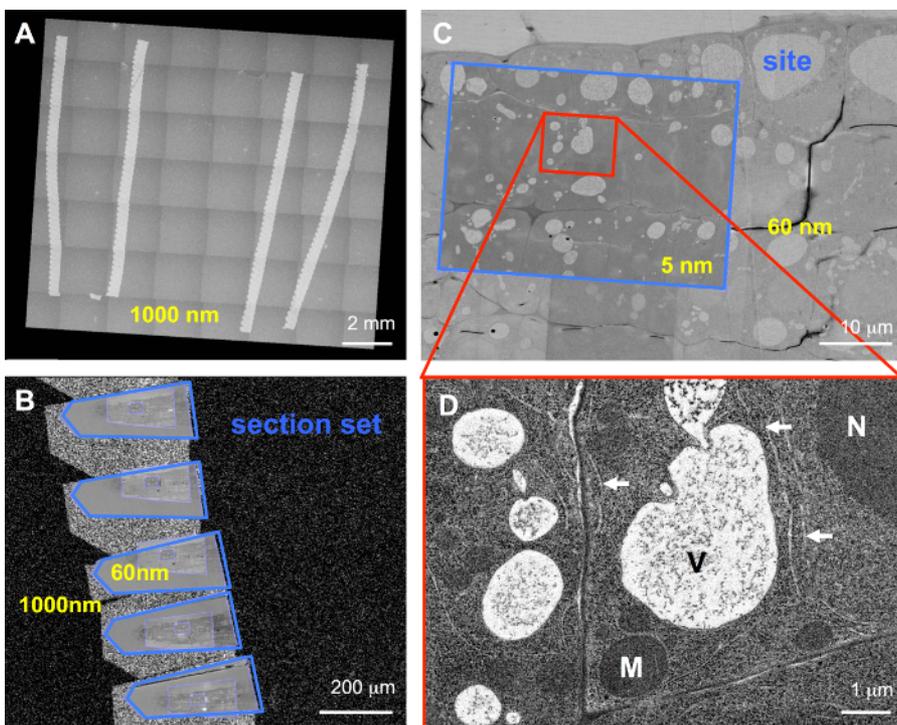

Figure 4: Hierarchical imaging in the SEM using ZEISS Atlas 5 AT. Overview of an array recorded with 1,000 nm image pixels using the SE detector (**A**). Section sets with the ROI placed on tissue in every section and recorded with 60 nm image pixels (**B**). Site sets with a serial ROI placed on target cells and recorded with 5 nm image pixels (**C**). When zooming into such high-resolution images (**D**), intracellular membrane compartments such as vacuoles (V), nucleus (N), mitochondria (M), and endoplasmic reticulum (arrows) become visible. Please click here to view a larger version of this figure.

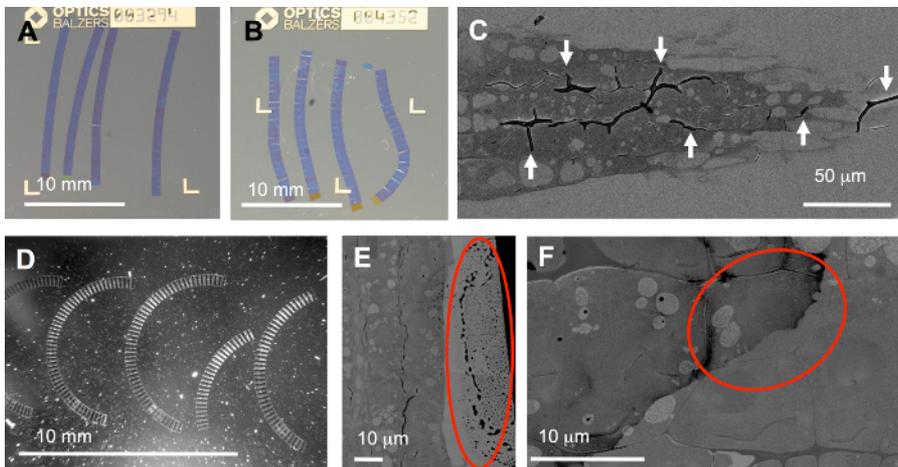**Figure 5: Typical problems.** 1. Arising from the sectioning process: Ribbons placed on ITO-coated coverslips are ideally straight (**A**), but irregular compression during sectioning may cause bent (**B**) or curved ribbons (**D**), or even folds (**C**). 2. Caused by handling substrate and ribbons in water, *e.g.*, during sectioning and staining: Light scattering particles on substrate (**D**), rim of droplets on section (circle in **E**), or dirt smeared out over tissue due to inadequate washing after staining (**F**). Please click here to view a larger version of this figure.

Supplemental Movie S1: FLM image stack. 435 images aligned in Fiji[16] using TrakEM[17] and saved as movie file (.avi). Please click here to download this file.

Supplemental Movie S2: SEM image stack. 210 images aligned in Fiji[16] using TrakEM[17]. The original stack (300 images) of this data set was 15 GB. To downsize the stack from 3.3 GB (after alignment and cropping to only the two target cells), it was scaled in x and y by a factor of 0.2 using Fiji and then saved as .avi movie. Please click here to download this file.

Supplemental Movie S3: Zooming with different resolution levels in the SEM. Movie created in and exported from the Atlas 5 software in .mp4 format. Please click here to download this file.

Discussion

A workflow for targeting specific cells within a tissue by multi-modal hierarchical AT was demonstrated: A resin-embedded sample is sliced up into arrays of serial sections, which are placed on a conductive substrate using a custom-designed substrate holder. After labeling with a fluorophore and imaging in the FLM, the reconstructed volume is used for selecting the target cells. After additional staining rounds with heavy metals to introduce contrast, these targets are imaged over several hundred sections at nanoscale resolution in an SEM using an automated software platform.

For producing densely packed arrays with several long ribbons, a substrate holder similar to the one described here is necessary. A skilled and patient person may be able to attach several ribbons to a silicon substrate, semi-immersed in the knife boat, and retrieve the array by gradually lowering the water level until the ribbons are sitting on the substrate. However, in the our experience, there is a tendency to shatter formation when the substrate is touching any part of the knife boat (cf. note in 1.3.2 in protocol). In addition, this procedure is much more difficult with ITO-coated substrates: (1) due to the transparency of the ITO-glass, it is difficult to see the edge of the water where the ends of the ribbons have to be attached; and (2) because the ITO-coated surface is much rougher than the highly polished silicon wafer, the ribbons tend to break during lift-out and smaller fragments consisting of a few sections may float, thus destroying the order of the sections.

The whole workflow is also feasible without correlation to FLM data. In this case, data collection in the SEM may have to be performed in several sessions. An initial 3D reconstruction or at least evaluation of low or medium resolution data may be necessary to identify targets. In addition, conventional histological stains for brightfield LM (not requiring FLM) may be applied. Of course, other options[6,7,8] are antibody labeling on the arrays, as already demonstrated in the initial paper on AT[18], or genetically encoded fluorescent proteins (XFPs) or pre-embedding labeling with preservation of fluorescence during sample preparation.

A general limitation of the discussed method is the use of sections of a certain thickness and the resulting discrete sampling of the 3D volume: Resolution in Z can only be as good as the thickness of the sections since the SEM collects only data from the section surface (depending on the primary energy/landing energy selected). This means that the resulting 3D volume has anisotropic voxels, *e.g.*, $5 \times 5 \times 100$ nm^3 if 100 nm sections and an image pixel size of 5 nm are used. For very small entities in a size range below 1 μm, this may not be sufficient for a true ultrastructural description. A more technical limitation is the accuracy of the stage used in the SEM for automated imaging. Due to this, it is necessary to choose an ROI larger than the specifications of the stage accuracy to guarantee that the full target area is imaged.

Compared to SBF-SEM and FIB-SEM as block-face imaging methods, correlative AT has the definitive disadvantage of anisotropic voxels, as described above. With FIB-SEM, isotropic voxels of $5 \times 5 \times 5$ nm^3 can be obtained when a proper drift correction is in place.

Gaps in the reconstructed volume due to loss of sections during preparation of arrays might also be a concern that is not encountered with SBF-SEM or FIB-SEM. With good ribbon stabilization by glue, this usually is only an issue for the last section of a ribbon: It might be damaged when releasing it from the knife-edge using the eyelash. However, in our experience, the loss of one section in every 20–50 sections does not influence image registration.

On the other hand, the possibility to post-stain arrays confers good signal and contrast for SEM imaging, even on weakly metalized samples such as the high pressure frozen root tips shown here. Therefore, it is not necessary to compromise optimal ultrastructural preservation by numerous chemical fixation and metallization steps. Also, routine samples from the pathology lab with intermediate degrees of metallization deliver excellent data[10]. Such a post-embedding contrast enhancement is not possible for SBF-SEM and FIB-SEM in general. Furthermore, since these methods are destructive, *i.e.*, consuming the sample during imaging, hierarchical imaging at different resolutions and sites or repeated imaging at later points in time is impossible. In principle, unlimited volumes, consisting of large FOVs (*e.g.*, up to several millimeters for whole mouse brains in connectomics) created by stitching mosaics, and huge numbers of sections can be acquired by AT, while in FIB-SEM, FOVs beyond 100 μm x 100 μm are difficult to achieve with routine instruments.

Further automation of the described AT-workflow would be a definite advantage, since the above-mentioned methods SBF-SEM and FIB-SEM perform both sectioning and imaging within the same instrument in a fully automated manner. One kind of automation of sectioning exists: The ATUMtome[12] can generate and collect thousands of sections, but the use of Kapton tape as a substrate makes such arrays difficult to image in a FLM. On the ITO-coated coverslips used here, even super-resolution imaging should be possible. A further, very desirable target for automation would be the recording of the FLM data stacks. On the other hand, automation can be expensive and except for the substrate holder, the workflow presented here relies (in terms of hardware) only on instrumentation usually available in a routine EM laboratory or core facility, making it low level access.

Disclosures

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