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# 2D and 3D Electron Microscopy (EM) Imaging of Tissue Biopsies and Resections

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## ABSTRACT

Recent developments in large format electron microscopy have enabled generation of images that provide detailed ultrastructural information on normal and diseased cells and tissues. Analyses of these images increase our understanding of cellular organization and interactions and disease-related changes therein. Here, we describe a workflow and generalized conditions for two-dimensional (2D) and three-dimensional (3D) imaging, including optical using Toluidine-Blue stained sections, large-format high-resolution scanning electron microscopy (SEM) montages, and 3D focused ion beam-scanning electron microscopy (FIB-SEM) image stacks, that allow pathologists and cancer biology researchers to identify areas of interest from human cancer biopsies.

Note: Imaging conditions described here may differ somewhat depending on microscope manufacturer. Efforts have been made to generalize for any model, but small changes may be necessary. OHSU's primary microscope for this work is a FEI Helios NanoLab G3 DualBeam FIB-SEM.

## EXTERNAL LINK

<https://doi.org/10.1016/bs.mcb.2020.01.005>

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Riesterer JL, López CS, Stempinski E, Williams M, Loftis K, Stoltz K, Thibault G, Lanicault C, Williams T, Gray JW. A workflow for visualizing human cancer biopsies using large-format electron microscopy. *Methods in Cell Biology*. 2020; doi:10.1016/bs.mcb.2020.01.005

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## KEYWORDS

scanning electron microscopy, FIB-SEM, 3DEM, high resolution EM montages

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GUIDELINES

Whenever handling samples, sample mounts, or working inside the microscope, wearing gloves is essential. Hand oils will contaminate the microscope vacuum and degrade imaging performance.

SAFETY WARNINGS

Electron microscopes operate at high voltage using high- and ultra-high vacuums. Follow manufacturer safety guidelines.

Diamond knives for ultramicrotomy are extremely sharp and delicate. Excise caution during use.

### Toluidine blue (TB) screening

- 1 Once the samples have been processed for 3DEM using a preferred protocol, sections from the resin block can be stained with Toluidine Blue. Our preferred protocol is published here:



Jessica Riesterer, Erin Stempinski, Claudia Lopez. Post-Fixation Heavy Metal Staining and Resin Embedding for Electron Microscopy (EM).

<http://dx.doi.org/10.17504/protocols.io.36vgre6>

This step allows researchers and pathologists to decide if more sectioning is needed to expose areas of the sample that are deeper within the tissue, providing at the same time information regarding tissue quality and heterogeneity. The TB section obtained using this method encompasses the entire block face, the acquired optical image can be easily overlaid with the EM map image if correlation is needed.



Sridharan G, Shankar AA (2012). Toluidine blue: A review of its chemistry and clinical utility.. Journal of oral and maxillofacial pathology : JOMFP.

<https://doi.org/10.4103/0973-029X.99081>

- 1.1 The entire block face (~1 mm<sup>2</sup>) is sectioned using an ultramicrotome and a diamond histology knife to generate 650 nm sections that are floated onto water. The final section(s) from the block is collected using a loop and placed onto a glass slide.
  - 1.2 Dry the glass slide to secure the sections on a hot plate at ~70°C for 5 minutes.

Place 1-2 droplets of Toluidine Blue stain on the dried sections. Set slide with stain droplet back onto

- 1.3 the 70°C hotplate for 10-12 seconds. Rinse stain with diH<sub>2</sub>O until runoff is clear.
- 1.4 Dry the glass slide again on a hot plate at ~70°C for 5 minutes to dry any remaining liquid.
- 1.5 Sections can be reviewed under a standard light microscope or digitized using a slide scanner.

#### Sample stub preparation for large format mapping and 3DEM

- 2 Preparing a resin block face for large format 2D mapping and 3DEM via FIB-SEM is straight-forward. This workflow works best when the Toluidine Blue screening sections described in step 1 are the last consecutive sections coming from the mounted block face. The mounting pins described below can be used on a standard microtome and allow adequate diamond knife clearance. As a result, Toluidine Blue sections could be produced after sub-step 2.1, allowing for correlation between the digitized section image and the EM map.
  - 2.1 Blocks produced for EM are mostly empty resin and too tall to be inserted into the SEM as processed. Using a razor blade the empty resin surrounding the stained tissue should be removed. The resin and tissue are very brittle, so leaving some excess empty resin is fine. This can be removed in sub-step 2.3.
  - 2.2 Blocks rough trimmed from the empty resin are **mounted** on Microtome stub SEM pins (Agar Scientific, cat# 61092450) or Aluminum Mini Pin SEM pins (Ted Pella, cat# 16180) using conductive silver epoxy (Ted Pella, Inc., H2OE EPO-TEK® cat# 16014). This is a two part epoxy that is mixed equally and cured using the manufacturer's recommended time and temperature schedule.
  - 2.3 **Fine trimming** of the mounted blocks is done using an ultramicrotome. The block should be trimmed using a diamond histology knife to create a smooth surface with few or no scratches. The block face needs to be as close to parallel to the SEM stub as possible to help the autofocus routine during imaging. Therefore, the ultramicrotome should have all of the angles set to 0°, except for the appropriate knife clearance angle. Additional trimming to tidy the block sides using a Diatome trim90 diamond knife into a 500x500x500 µm<sup>3</sup> pillar can be performed, but is not necessary. However, if an excess of empty resin still surrounds the tissue after sub-step 2.1, this should be removed with a trim tool. The empty resin will produce extra charging under the electron beam.
 

NOTE: if standard 12 mm SEM stubs are being used, trimming and sectioning directly from a resin-embedded BEEM capsule or coffin mold formed block is advised prior to stub mounting, as the diamond knife will most likely collide with the larger diameter SEM stub.
  - 2.4 **OPTIONAL:** The edges of the resin block should be painted with conductive silver paint (Ted Pella, cat# 16035) to aid in charge mitigation. Care should be taken such that the newly trimmed block face is not painted. Be sure to shake the paint before use. Allow to dry for 20 minutes at room temperature.
  - 2.5 **Conductive coating** is necessary to achieve high-resolution, high contrast, low noise images on samples that build up surface charge while imaged with an electron and/or ion beam. Carbon is preferred for FIB-SEM due to its amorphous nature, minimizing ion milling artifacts (i.e., curtaining) that distort final image quality. However, metal coatings (i.e. irridium, gold, platinum, etc.) can be used if carbon is not available. Suggested coating thicknesses are 8-nm for carbon or 4-nm for metal. The ideal thickness will provide adequate charge mitigation under the electron beam, but will not be so thick that it is obviously visible in images.

A Leica ACE600 High Vacuum Sputter Coater fitted with a rotating platform is used in the OHSU laboratory. The coating thickness is monitored and measured with an *in situ* quartz crystal localized in the center of the rotating platform.

## EM Imaging

- 3 Once a sample preparation protocol is established, imaging conditions for the respective 3DEM technique typically will not vary much for the same tissue or cell type. Depending on the exact microscope model, small variations will be needed from those reported here. While collecting a stunning image for analysis can be straightforward, image collection parameters must also be optimized for post-imaging data handling. Scanning conditions chosen here provide the best membrane contrast and signal-to-noise ratio, while preventing charging artifacts and being conscientious of data collection time.

- 3.1 Sample stubs should be loaded into the microscope as per the microscope manual. Allowing the chamber to reach high vacuum prior to imaging is recommended.

### 3.2 Large Format 2D Mapping

The Thermo Scientific Maps™ software for SEMs allows high resolution imaging (4 nm/pixel) across the entire block face by montaging hundreds of images together during automated image tile acquisition. The resulting high-resolution map has panning and zooming capabilities and can be used like “Google Street-View” for the SEM. Stage positions are saved within the project, allowing specific ROIs identified earlier to be found with ease even if the sample has had to be moved from the microscope in the meantime. Similarly, the Zeiss Atlas software can be used on a Zeiss Crossbeam FIB-SEM.

Typical imaging conditions used for data collection on an FEI Helios NanoLab G3 DualBeam™ FIB-SEM are 2-3 keV, 200-400 pA, 4 mm working distance with the retractable directional backscattered (DBS) electron detector. Tiling is done using 10% overlap in both *x* and *y* directions. Typical imaging conditions on a Zeiss Crossbeam 550 for similar imaging quality would be 1.0 keV, 1.5 nA, 5 mm working distance with the Energy Selective Backscatter (EsB) detector.

It is recommended to collect a low-resolution overview map prior to high resolution mapping to ensure the block is good enough to invest the microscope time into. A suggested lateral pixel resolution is 40-nm will be sufficient to assess fixation and processing quality. Data collection time for this is roughly 1-hour.

Large-format high-resolution montages are acquired over 15-24 hours depending on size of area being acquired (~500 x 500 μm<sup>2</sup>). Lateral pixel resolution is 4-nm for high resolution montages. Individual images are stitched together collection using the vendor software algorithms, in-house algorithms, or a freeware package, such as FIJI.

Digitized TB section images are imported as tiff files and overlaid onto the EM montage via Maps or Atlas software-driven 3-point alignment procedure. The correlated images can be faded into each other by opacity adjustments, also providing aid in locating ROIs.

### 3.3 High Resolution 3DEM via FIB-SEM

The FIB-SEM is also used to collect targeted 3D volumes using a heated Ga<sup>+</sup> liquid metal ion source (LMIS) slicing 4nm-thick for hundreds of slices. The slicing/imaging cycle is automated using the Thermo Fisher Scientific AutoSlice and View™ or Zeiss Atlas software packages. There is no need to remove the sample from the microscope before 3D data acquisition, but if necessary a three-point alignment using the montage created in step 2 and the current stage position is recommended in order to easily find ROIs.

Samples are tilted such that the block face is perpendicular to the FIB (52° on a FEI/Thermo Scientific instrument, 54° on a Zeiss or Tescan) for thin slicing. Without moving the stage, a backscattered electron image is generated with the SEM after slicing is completed. The process repeats to form an image stack which is later aligned and annotated. Fiducial markers are placed near the ROI to aid in

slice and image placement. A carbon or platinum protective capping layer is deposited over the volume to be collected in order to maintain the sample integrity during ion beam fiducial recognition.

Imaging and slicing conditions for FIB-SEM data generation in this workflow are found standard on most instruments. Imaging conditions will be the same as used in step 2. During 3D data collection on a FEI/Thermo Scientific Helios FIB-SEM, the in-column (ICD) and through-the-lens (TLD) detectors are used to form backscattered electron images. The TLD is used for fiducial recognition and automated alignment functions. The ICD provides the final image used in data reconstruction due to its superior ability to image fine, crisp membranes with higher contrast. Milling conditions are 30 keV, 0.79 nA with a 30- $\mu\text{m}$  z-depth. Typical milling conditions on a Zeiss Crossbeam 550 are 30 keV, 0.7nA with a 30- $\mu\text{m}$  z-depth. Images are collected using the EsB detector.

FIB-SEM provides roughly  $25 \times 20 \times 8 \mu\text{m}^3$  volumes in 72 hours. Voxel sizes are isotropic at 4 nm resolution. These volumes are suitable for viewing cellular components like mitochondria, Golgi apparatus, and nucleoli in fine detail.

Once completed, image stacks are exported to a third-party software, such as Thermo Scientific Amira or ORS Dragonfly, for registration and segmentation.