Spin Mill Bio Method for large-area planar milling

Accessing large areas and 3D imaging with the Hydra Bio Plasma-FIB

Combined focused ion beam scanning electron microscopy (FIB-SEM) is a key technology in the life sciences and has become a common method for 3D analysis of biological samples at high resolution. Plasma-FIB (PFIB) SEM tomography is based on the cross-section technique, where the sample is perpendicular to the ion beam and remains tilted during the imaging process.

In this application note, we describe a new approach, the the Thermo Scientific™ Spin Mill Bio™ Method, for large-area planar milling with oxygen PFIB for accessing and investigating large areas (up to 1 mm). The Spin Mill Bio Method on the Thermo Scientific™ Hydra Bio™ Plasma-FIB can offer an alternative approach to 3D analysis for biological samples, expanding the PFIB-SEM application beyond the conventional cross-section technique.

The Spin Mill Bio Method is based on removing a thin layer from the sample surface at a nearly glancing angle, which can also be described as a method to polish horizontal planes. A broad area up to 1 mm in diameter can be irradiated with a plasma ion beam using xenon, argon, nitrogen or, most commonly, oxygen in life science resin-embedded samples at a glancing angle. Commonly used angles are between 1° and 5°, but most often 4° (**Figure 1A**). The stage is periodically rotated to a series of pre-defined milling sites referred to as Spin Mill position. The number of milling positions can contribute to optimized quality of the sample surface. Most commonly, 5 milling positions are used. A full rotation of 360°under the ion beam constitutes a single milling slice.

Milling one slice can take a few seconds up to a few minutes, based on the diameter of the milled area and milling parameters such as high voltage, beam current, and slice thickness. For example, one slice with 10 nm thickness and an area 240 µm in diameter takes 30 seconds to mill using oxygen ion beam (12 kV, 64 nA).

This milling method does not require preparation steps such as trenches, fiducial marker, or protective layer deposition. This significantly reduces the time required for the entire experiment. Preparing a selected area for the Spin Mill Bio Method using a circular marker around the area of interest for orientation and accurate definition of Spin Mill positions. This circular marker is typically used when the area of interest is smaller than the sample surface, or when the entire sample's surface is larger than 1 mm in diameter (**Figure 2A**). It is also possible to spin mill the whole sample surface without creating a circular maker if the sample surface is relatively small (e.g. 189 µm, as shown in **Figure 1**). In this case the entire sample surface is considered an area of interest.

Once the full rotation is completed, the stage automatically tilts back to a 0° angle (**Figure 1C**), with the electron beam perpendicular to the sample surface for acquisition of the SEM images (**Figure 1D**). Thin slices in the nanometer range can be automatically milled and imaged iteratively, like in serial block-face imaging. This results in a high-resolution 2D-image stack that can be combined for 3D visualization of features of interest at nanoscale resolution.

The Spin Mill Bio Method is fully automated and easy to set up using Thermo Scientific™ Auto Slice & View™ Software (Figure 3C). Multiple areas for image acquisition within one Spin Mill Bio experiment can be selected (**Figure 2**). Each area of interest can be imaged at different imaging settings based on the specificity of the experiment (**Figures 3B** and **4**).

The Spin Mill Bio Method, in combination with the flexibility of multi-ion milling, offers new possibilities for accessing large areas previously considered challenging in a wide range of life-science samples. This application is especially beneficial for identifying and accessing sparse regions of interest easily, as well as generating quantitative data for statistical validation[1] or the characterization of feature distribution and morphology in different cell populations or experimental conditions.

1. Li S., Raychaudhuri S., Lee S.A, et al. (2021) *Asynchronous release sites align with NMDA receptors in mouse hippocampal synapses.* **Nature Communications** 12:677. **Doi: 10.1038/s41467-021-21004-x**.

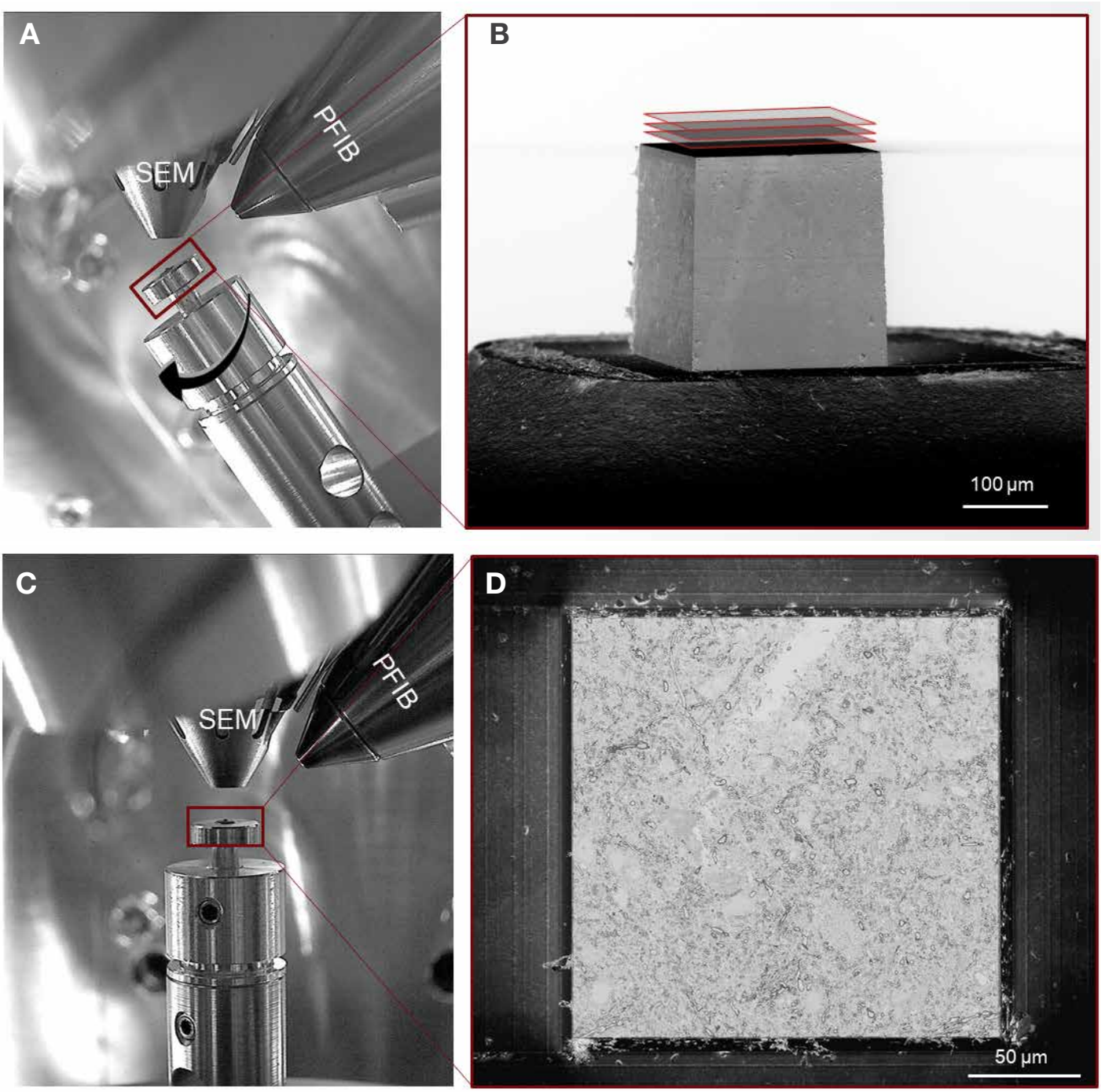

Figure 1. PFIB-SEM Spin Mill Bio Method: geometry of PFIB and SEM in respect to the sample. (A) Position of the stage and sample during the milling process. The stage is tilted to negative 34° with the ion beam at a 4° angle to the sample surface. The stage continuously rotates by a defined number of Spin Mill positions, during which the sample surface is exposed to the ion beam. (B) Ion beam view under the 4° angle. The sample in fifth Spin Mill position with rotation stage at 288°. This image illustrates the sequential milling of the entire sample surface (189 x 187 µm) in each Spin Mill position. (C) The stage with the sample at imaging position (0° tilted) with the sample surface being perpendicular to the electron beam. (D) Mouse brain tissue embedded in EPON epoxy resin. The figure shows a sample surface during the imaging process, when the freshly milled sample is imaged after each slice is milled.

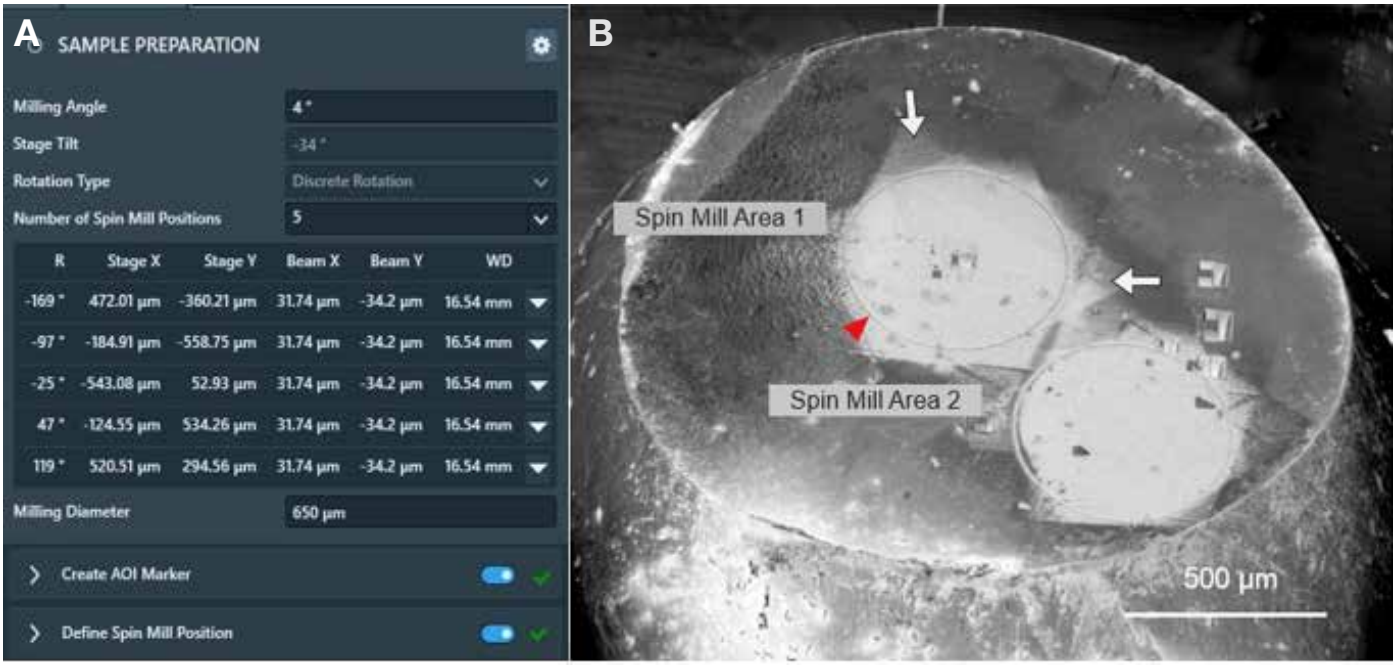

Figure 2. Preparation of the sample for Spin Mill Bio Method. (A) Setting the Spin Mill area in sample preparation mode of Auto Slice & View 5 Software. The area of interest represents the area that will be spin milled. The milling angle, milling diameter, and number of Spin Mill positions with their stage coordinates are set in the application. The coordinates of Spin Mill positions result from the "Define Spin Mill Position" step, which automatically generates the coordinates for each position. The parameters shown in this example depict the preparation of Spin Mill Area 1 with a milling angle of 4°, five Spin Mill positions, and a milling diameter of 650 µm in the software interface. (B) The sample surface with two Spin Mill areas is defined by a circular area of interest (AOI) marker (red arrow). The milling process results in a starlike pattern on the sample surface that represents milling traces made at each Spin Mill position (white arrows).

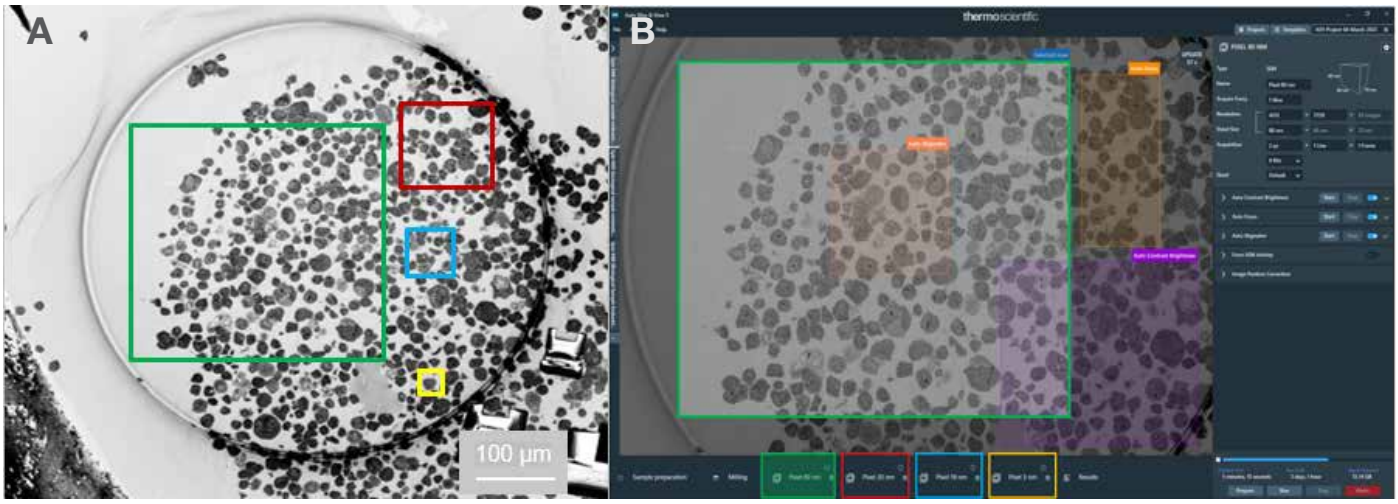

Figure 3. Setting the image acquisition parameters of Spin Mill Bio area in imaging module of Auto Slice & View 5 application. (A) Detail of the Spin Mill Area 2 (see Figure 2), marked by a circular area of interest marker. Four different AOIs are selected for the image acquisition, represented by colored rectangles. (B) Overview of the image acquisition settings, including auto functions (auto contrast brightness, auto focus, auto stigmator) applied for the first AOI (green rectangle). In this example, an 80 x 80 x 70 nm voxel size with 4,312 x 3,726 pixel resolution was selected with a 2 µm dwell time and line frame integration 1. The image acquisition settings for all the other areas of interest, represented by red, blue, and yellow rectangles, are different (details shown in Figure 4). Image acquisition settings for each area of interest are set in a separate page within the Imaging module of the Auto Slice & View 5 workflow.

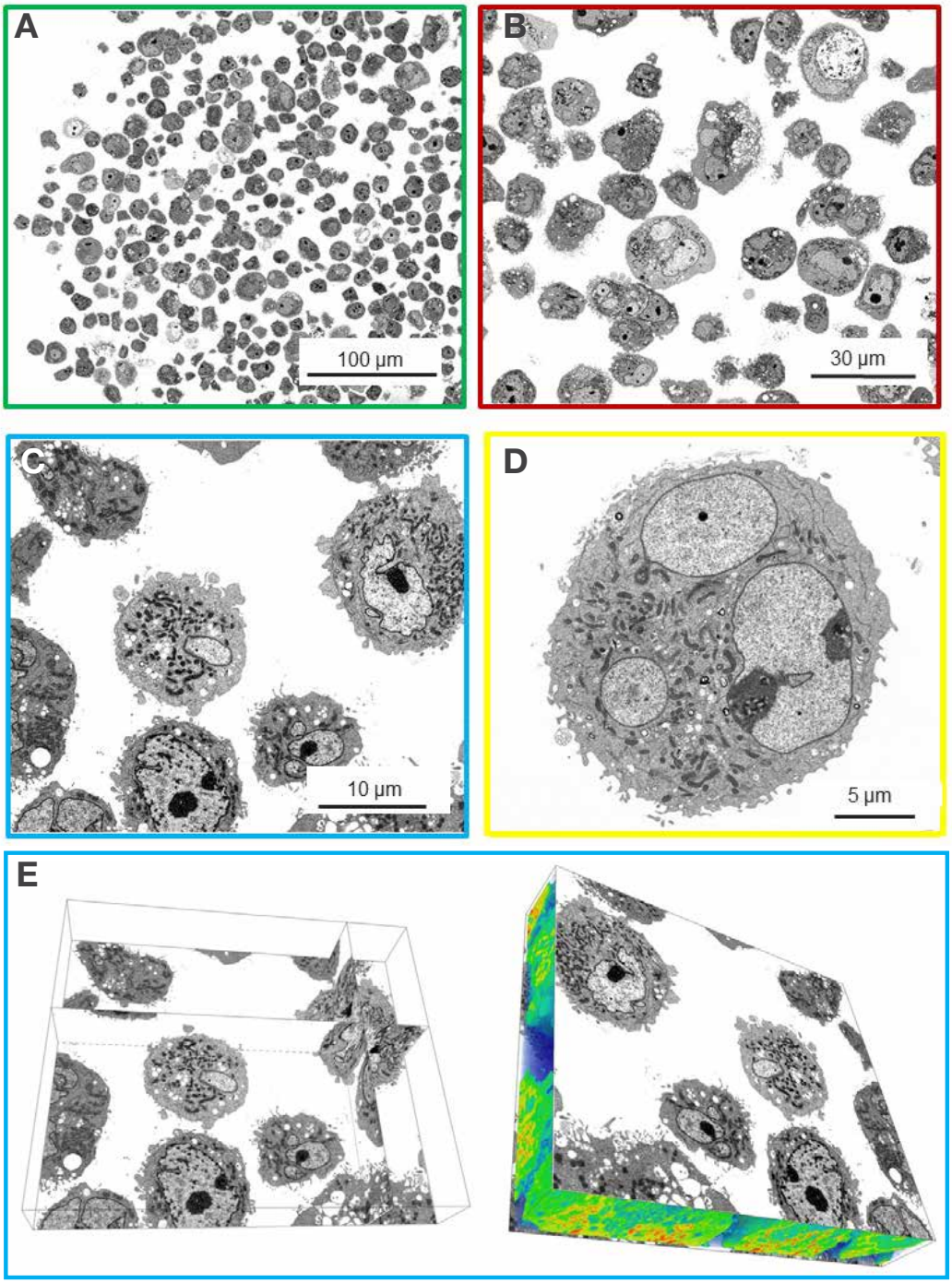

Figure 4. A selected slice for each area of interest (AOI) from the Spin Mill Bio PFIB-SEM image stack. Imaging was done with CBS detector at 2 kV and 200 pA for all examples. (A) AOI 1 with horizontal field width of 354 µm and 80 x 80 x7 0 nm voxel size (4,312 x 3,726 pixel resolution, 2 µm dwell time). (B) AOI 2 with horizontal field width of 138 µm and 30 x 30 x 70 nm voxel size (4,604 x 3,974 pixel resolution, 2 µm dwell time). (C) AOI 3 with a horizontal field width of 44 µm and 10 x 10 x 70 nm voxel size (4,408 x 3,689 pixel resolution, dwell time 2 µm). (D) AOI 4 with horizontal field width of 25 µm and 3 x 3 x 70 nm voxel size (8192 x 7485 resolution, dwell time 1 µm). (E) Sample volume of region of interest 3 with selected slices along the XY, ZX, and ZY planes with 3D reconstructed volume of ~44 x 37 x 8 µm. Total time for experiment ~6.5 hours (1.5 hours of milling and 5 hours of image acquisition of all four AOIs). Chinese hamster ovarian cells embedded in DURCUPAN resin. *Sample courtesy of Core Facility Cryo-electron Microscopy and Tomography of CEITEC Masaryk University.*

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