

Step-by-step procedure for an optimized serial lift-out cryo-focused ion beam milling technique in tissue analysis

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Abstract Cryo-focused ion beam (cryo-FIB) milling is indispensable for preparing thin lamellae from thick biological samples for cryo-electron tomography (cryo-ET), especially at the tissue level. However, existing approaches remain technically demanding and susceptible to lamella instability. We present a detailed, optimized serial lift out cryo-FIB milling protocol specifically designed for multicellular tissue samples, emphasizing improved lamella stability, reproducibility, and reduced operator dependency. This method integrates six-sided attachment geometry and adjustable lamella slit spacing, enabling efficient lift-out and precise reattachment of variable-sized tissue volumes to pre-milled grids. The procedure is applicable across a range of biological tissues and is compatible with integrated cryo-correlative light and electron microscopy (cryo-CLEM) pipelines. By enhancing structural preservation and simplifying operational steps, this protocol provides a robust, reproducible framework to facilitate high-throughput, *in situ* structural analysis of tissues by cryo-ET.

Keywords Cryo-focused ion beam (cryo-FIB), Cryo-electron tomography (cryo-ET), Serial lift out, Lamella stability, Multicellular tissue samples

INTRODUCTION

Cryo-ET has revolutionized the visualization of macromolecular structures within their native biological environments, enabling the integration of molecular, cellular, and tissue-level organization at near-nanometer resolution (Baumeister 2022; Berger *et al.* 2023). Although cryo-ET preserves ultrastructural integrity with exceptional fidelity, its application to thick, multicellular specimens – such as mammalian tissues – requires extensive thinning to achieve electron transparency. Cryo-FIB milling has become an essential technique for preparing lamellae suitable for

cryo-ET from such samples (Schiotz *et al.* 2024b). Various approaches – including the Waffle method (Kelley *et al.* 2022), ultramicrotome trimming combined with cryo-FIB (Zhang *et al.* 2021), on-grid milling (Wang *et al.* 2023), and Serial Lift-Out (Schiotz *et al.* 2024a) – have been developed to overcome the challenges associated with sample thickness. However, current cryo-FIB workflows, especially those involving lift-out procedures, often require highly trained operators and face limitations such as low reproducibility, lamella breakage, and misalignment.

To overcome these challenges, we present an optimized serial lift out cryo-FIB protocol specifically tailored for tissue samples. Our method incorporates a six-sided attachment strategy and adjustable grid trench with plateaux configurations, which together significantly enhance the mechanical stability of lamellae and reduce dependence on operator expertise. This

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protocol enables rapid lift-out and precise repositioning of large tissue volumes, facilitating the efficient production of multiple lamellae from a single sample block while minimizing iterative adjustments.

We have successfully applied this method to mouse intestinal tissue, generating high-quality lamellae suitable for cryo-ET imaging of microvillar structures and their underlying actin cytoskeleton. Here, we provide a detailed, step-by-step description of our optimized serial lift out cryo-FIB milling workflow. Designed to be accessible to users with intermediate cryo-FIB experience, this protocol aims to standardize lamella preparation across laboratories, thereby broadening the applicability of cryo-ET in the study of complex biological tissues.

Development of the protocol

The protocol commenced with cryo-preserved mouse intestinal tissue samples embedded in high pressure freezing (HPF) carriers. The optimized serial lift out workflow was systematically divided into four main stages: sample block lift-out, receiver grid preparation, six-sided attachment, and fine milling. In summary, a receiver grid was first milled to create defined slits with plateaus. Subsequently, a sample block containing an intestinal villus segment, previously localized using cryo-CLEM, was detached and lifted out using an EasyLift manipulator. The sample block was then repeatedly attached to and released from the slits on the prepared grid. Finally, fine milling was performed to produce 200 nm-thick lamellae. This procedure ensures reliable lamella generation with the volume of interest (VOI) suitable for users with minimal training.

Applications and advantages of the protocol

The optimized serial lift out protocol, demonstrated using mouse intestine as a model, is broadly applicable to various tissue types for the routine generation of lamellae suitable for users at different experience levels. The protocol provides four primary advantages. First, the lamellae are secured on all six sides to the receiver grids, ensuring enhanced structural stability and minimizing the risk of breakage or loss during loading into cryo-electron microscopes. Second, the plateaus on the receiver grids designed for lamellae attachment are adjustable, accommodating sample blocks of varying widths and thereby increasing adaptability to differently sized samples. Third, the six-sided attachment approach reduces the necessity for precise control of either the EasyLift manipulator or the ion beam milling patterns, effectively lowering the technical skill requirements for users. Furthermore, the workflow is designed not only to extract the sample

block from the top surface of the sample carrier but also from the horizontally milled trench directions within the sample carrier, as illustrated by J. Liu *et al.* (Liu *et al.* 2025).

Limitations of the protocol

The protocol still requires users to have received basic training in cryo-FIB milling techniques.

Overview of the protocol

As shown in supplementary Movie S1, the intestinal villi were initially correlated with CLEM to define the VOI. Subsequently, the sample block at the VOI was lifted out using the EasyLift manipulator. The sample block was then attached to the plateaus of pre-prepared receiver grids using a six-sided attachment approach. Following this, the first lamella was released from the sample block by milling a line pattern at approximately 4 μm above the lower edge of the sample block. The remaining portion of the sample block was then subjected to iterative sectioning to produce additional lamellae. Finally, fine milling was performed to generate thin lamellae suitable for data acquisition.

SUMMARIZED PROCEDURE

- 1 Fabricate receiver grids by milling trenches that incorporate two distinct plateaus.
- 2 Identify the VOI encompassing the intestinal villi using iFLM and MAPS software.
- 3 Extract a sample block from the selected VOI by carefully detaching it from the HPF block, followed by securing it onto the EasyLift manipulator.
- 4 Attach the sample block onto the two plateaus of the trench using a six-sided attachment method. Subsequently, detach a $\sim 4 \mu\text{m}$ section from the base of the sample block to generate the first lamella. Repeat this procedure to produce additional serial sections as needed.
- 5 Perform fine milling and polishing of all prepared lamellae to achieve the desired thickness.

EXPERIMENTAL DESIGN

High-pressure freezing

Intestinal segments ($\sim 1.5 \text{ mm} \times 1.5 \text{ mm}$, length $\times$ width) were isolated from two-month-old CAG-RFP-EGFP-LC3 transgenic C57BL/6NJ mice (JAX #027139)

as described by Weigmann *et al.* (Weigmann *et al.* 2007). Samples were placed in 3-mm diameter, 300 μm -deep HPF carriers (Leica) filled with 40% Ficoll in PBS, sealed with sapphire discs, and rapidly frozen using a Leica ICE system.

Receiver grids preparation

Three central 400-mesh bars of a G100/400 copper grid were milled to form trenches with dimensions of 40 μm $\times$ 40–50 μm $\times$ 10 μm (length $\times$ width $\times$ z-depth), 40 μm $\times$ 8 μm $\times$ 10 μm , and 40 μm $\times$ 40–50 μm $\times$ 10 μm , respectively, adjusted to accommodate the sample block. With the stage tilted at 7°, two cross-sectional patterns, each measuring 5 μm $\times$ 5 μm $\times$ 10 μm , were milled from both sides of the trench to create lamella attachment plateaux.

VOI registration by CLEM

The isolated intestinal villi were imaged using the iFLM correlative system with excitation wavelengths of 470 nm and 565 nm. The resulting fluorescent signals were then aligned with corresponding SEM and ion beam images using MAPS software, allowing precise localization of the intestinal villi and definition of the VOI.

Sample block lift-out

Trench milling was used to release the VOI on three sides with regular cross-section patterns, leaving one side unprocessed. The EasyLift manipulator was then attached to the top of the VOI via redeposition milling, after which the remaining side was milled to fully release the sample block for extraction.

Lamellae generation by six-sided attachment

The sample block was aligned above the plateaus and attached via redeposition from the sides and bottom. A $\sim$ 4 μm -thick lamella was released using line milling, and the remaining block was removed. The lamella was further stabilized by vertical redeposition at the top corners. This process was repeated until the VOI was fully sectioned, with all redeposition performed at 3 nA beam current.

Lamellae polishing

Fine milling is initiated with the removal of both the front and back surfaces of lamellae, followed by the deposition of a protective platinum layer. The milling

process was performed at a 7° stage tilt, starting with a notch pattern at 0.1 nA, followed by stepwise thinning at 1 nA (2.5 μm), 0.3 nA (1.5 μm), and 0.1 nA (0.8 μm). Final polishing was conducted at 30 pA, resulting in lamellae $\sim$ 200 nm in thickness.

PROCEDURE

High-pressure freezing preparation

- 1 Prepare a 40% (w/v) Ficoll 400 cryoprotectant solution in phosphate-buffered saline (PBS) buffer.
- 2 Coat the sapphire discs with a freshly prepared solution of vegetable lecithin (100 mg/mL in chloroform), as described by McDonald *et al.* (McDonald *et al.* 2010).
- 3 Cool down the ICE high-pressure freezer and fill the sample storage dewar with liquid nitrogen.

Mouse intestine tissue isolation

- 1 The intestines were isolated from two-month-old CAG-RFP-EGFP-LC3 transgenic C57BL/6NJ mice (JAX stock #027139), as previously described by Weigmann *et al.* (Weigmann *et al.* 2007).
- 2 Place the isolated intestinal tube in a 10-cm dish containing cold PBS, and gently rinse the intestine using a 1-mL pipette tip filled with cold PBS.
- 3 Longitudinally open the intestinal tube using fine scissors.
- 4 Cut a segment approximately 1 cm in length and transfer it to a new, sterile 10-cm Petri dish containing fresh cold PBS.
- 5 Using fine forceps, further dissect the intestinal segment into smaller fragments of approximately 1.5 mm $\times$ 1.5 mm.

High-pressure freezing

- 1 Obtain a Type B carrier equipped with a forceps and carefully place it into the 10-cm dish containing the isolated intestinal fragment.
- 2 Put the isolated intestinal fragment into the cavity of the type B carrier, ensuring proper orientation by positioning the intestinal villi facing upwards. Perform this procedure entirely within PBS buffer.
- 3 Carefully remove the carrier along with the intestinal fragment and transfer it onto a piece of filter paper.
- 4 Remove PBS buffer inside the carrier cavity using a sharpened tip of the filter paper.
- 5 Immediately apply a single drop of 40% Ficoll 400

solution into the carrier cavity containing the intestinal fragment.

- 6 Seal the carrier by covering it with a sapphire disc pre-coated with lecithin.
- 7 Place the assembled sandwich, consisting of the carrier, intestinal fragment, and sapphire disc, into the central aperture of the middle plate.
- 8 Securely assemble the two half-cylinders together with the middle plate containing the sample sandwich, and promptly transfer the assembly into the ICE high-pressure freezer.
- 9 Close the red lid of the freezer to initiate the automatic freezing cycle.
- 10 Repeat Steps 1–9 until all isolated intestinal fragments have been processed and frozen.
- 11 Retrieve the sample storage dewar from the Leica ICE high-pressure freezer and transfer all frozen samples into a liquid nitrogen dewar for long-term storage until further use.

Cryo-correlative light and electron microscopy (cryo-CLEM)

- 1 Cool down the Hydra 5 plasma FIB machine.
- 2 Transfer the frozen sample, housed within its

carrier, and load it into the left well of the P3 position on the P3/AG shuttle.

- 3 Place a clipped 100/400 mesh grid into the right well of the AG position on the P3/AG shuttle. Orient the mesh so that the 100-mesh bars are aligned vertically within the shuttle.
- 4 Perform sputtering and GIS coating on the frozen sample.
 - (A) Adjust the stage to the “sputter position” and deposit a layer of organic sputtering material by employing a polygonal milling pattern at a voltage of 12 kV and a beam current of 0.12 μ A (dwell time, 100 ns; milling time, 2 min).
 - (B) Move the stage to the “deposition position” and deposit a layer of platinum metal using the gas injection system (GIS) for a duration of 120 s.
- 5 [Optional] Conduct scanning electron microscope (SEM) imaging of the frozen sample.
 - (A) Go to “mapping position” of the sample carrier and focus.
 - (B) Acquire a high-resolution SEM overview image of the carrier using an exposure time of 2 μ s and an image size of 6144 $\times$ 4096 pixels at 13 pA.
- 6 Perform fluorescent imaging, as illustrated in Fig. 1.
 - (A) Adjust the eucentric positions of both the SEM

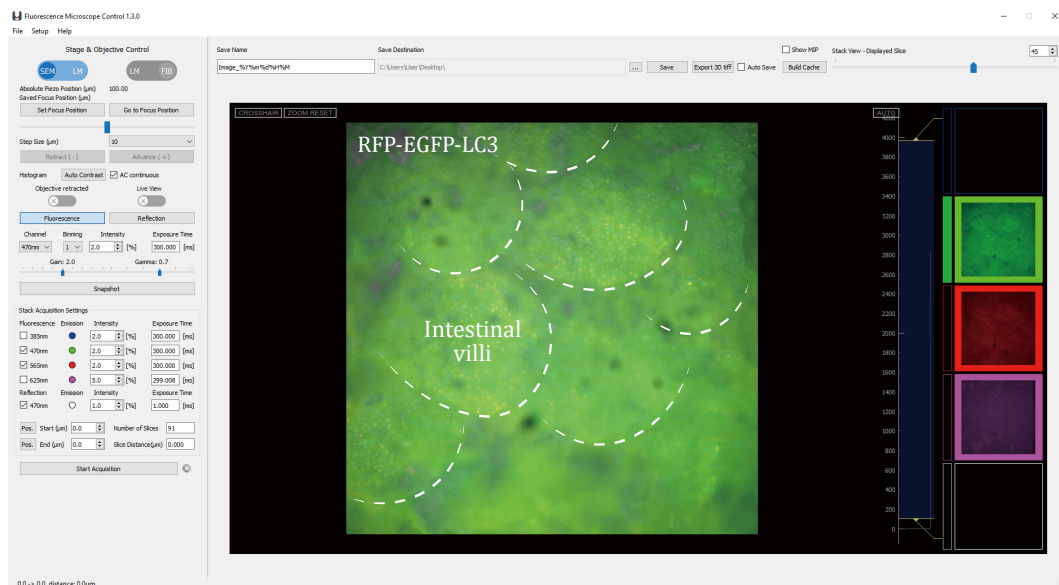


Fig. 1 Fluorescent and reflection stacks were acquired using iFLM software

- (B) Navigate to the “mapping position” and launch the iFLM software. Switch to the light microscopy (LM) position by selecting the “SEM/LM” button.

- (C) Focus the LM using the reflection light source.
- (D) Switch to fluorescent imaging mode with an emission wavelength of 470 nm and navigate across the sample carrier to locate the boundary of the frozen intestinal fragment.

- (E) Proceed to a defined edge of the intestinal fragment (*e.g.*, the upper left corner). Establish z-stacks and capture fluorescent images at 470-nm emission (for GFP-labeled structures), 565-nm emission (for RFP-labeled structures), and a bright image in reflection mode.
- (F) Progress to the subsequent imaging location and repeat Step E until the entire intestinal region has been imaged. Eight positions were imaged in total.
- 7 Correlate the SEM image with the iFLM fluorescent image stacks (Fig. 2).

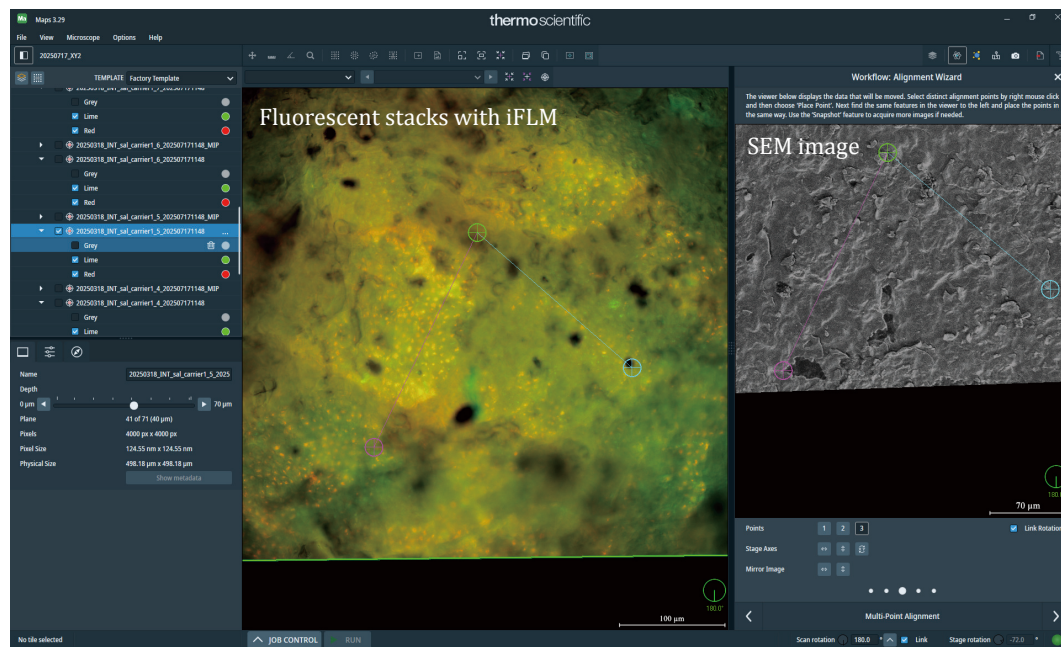


Fig. 2 The CLEM procedure was performed using MAPS software. The SEM image was aligned with the iFLM fluorescent image stacks using a three-point registration method

- (A) Add a new layer within the MAPS software.
- (B) Capture a snapshot of the carrier overview image.
- (C) Import the fluorescent image stacks into the MAPS software and adjust the contrast and brightness levels for each channel.
- (D) Based on the boundaries defined by the eight fluorescent stacks, delineate a tile area for acquiring the SEM image of the intestinal fragment.
- (E) Execute the tile acquisition using an exposure time of 2 μ s and an image size of 6144 $\times$ 4096 pixels at 13 pA, to generate a high-resolution SEM montage.
- (F) Identify a fluorescent stack of interest and acquire a corresponding SEM image using the same imaging parameters as outlined in the previous step.
- (G) Launch the alignment wizard within the software to initiate the registration process of the fluorescent stack.
- (H) Identify and mark three corresponding featured points, such as ice contaminations, in both the fluorescent stack and the SEM image.
- (I) Follow the guided steps provided by the alignment wizard to complete the alignment procedure.
- (J) Define the VOI based on the fluorescent signal distribution. A VOI measuring approximately 40 μ m $\times$ 50 μ m was selected.
- (K) Register the VOI position onto the correlated SEM image for further analysis.

Sample block lift out

- 1 Prepare the receiver grid (Fig. 3).
 - (A) Navigate to the center of the 100/400 mesh grid.
 - (B) Select three adjacent parallel vertical 400-mesh bars. At the center of each bar, generate a group of patterns with dimensions of 40 μ m $\times$ 40–50 μ m $\times$ 10 μ m (length $\times$ width $\times$ z-depth), 40 μ m $\times$ 8 μ m $\times$ 10 μ m, and 40 μ m $\times$ 40–50 μ m $\times$

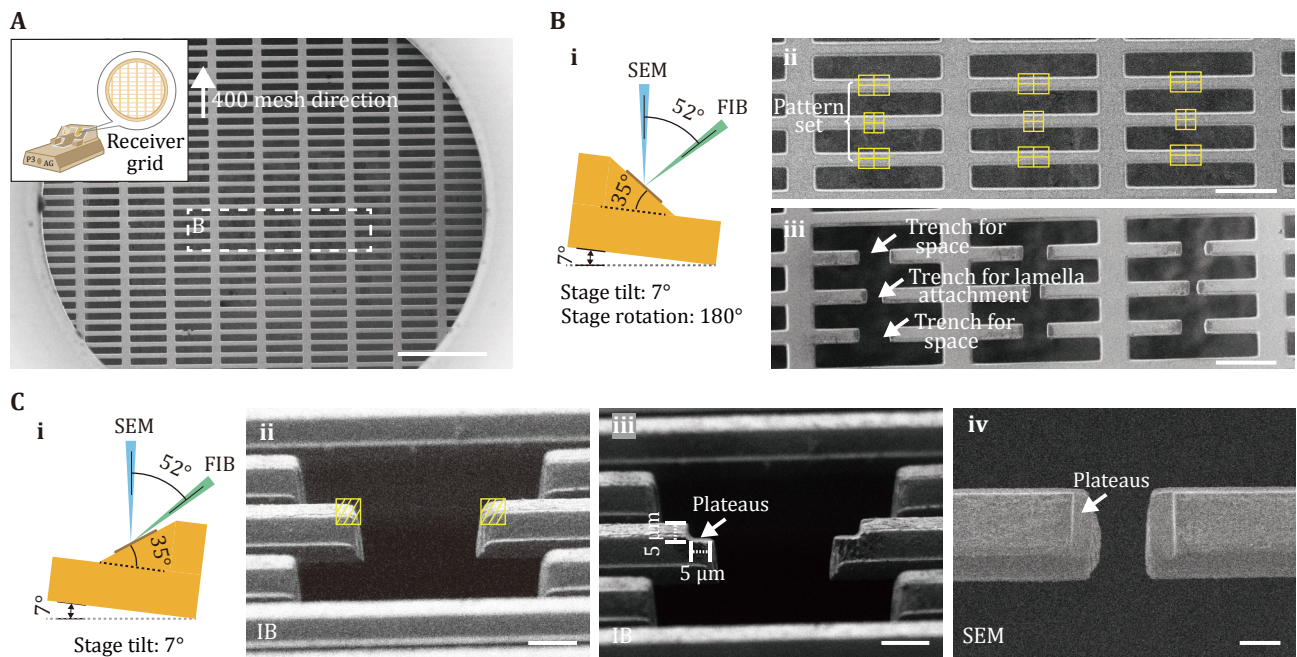


Fig. 3 Preparation of the receiver grid. **A** Locate the center of the 100/400 mesh grid and select three groups of adjacent and parallel 400-mesh bars. Scale bar = 500 μm . **B** Trench milling of the grid bars. (i) illustrates the stage position; (ii) shows the pattern sets used for trench milling; (iii) displays the grid bars after trench milling. Scale bars = 100 μm . **C** Fabrication of plateaus for sample block attachment. (i) Illustration of the stage position; (ii) shows the patterns used to create two plateaus; (iii) and (iv) present ion beam (IB) and scanning electron microscopy (SEM) images of the milled plateaus, respectively. Scale bars = 10 μm .

10 μm , respectively (Figs. 3A and 3B).

(C) Copy the group of three patterns and paste them onto other groups of three parallel grid bars as needed. Adjust the width of the middle pattern to match the width of your sample block. We have generated three groups of patterns as depicted in Fig. 3B. Specifically, the other two middle trenches are milled with widths of 10 μm and 18 μm , respectively.

(D) Mill these patterns at 30 kV and 60 nA to create grid trenches, which will take approximately 20 min with the Xenon source.

(E) Generate flat edges for each middle trench by milling with 15 nA using patterns of 1 μm in width.

(F) Rotate the stage by a relative 180° (stage tilt, 7°) to the milling orientation and save the position as “Receiver position”.

(G) Generate two plateaus by performing cross-sectional milling patterns (5 $\mu\text{m} \times 5 \mu\text{m} \times 10 \mu\text{m}$, 30 kV, 15 nA) at the edge of each middle trench (Fig. 3C).

2 Attach a copper block to the EasyLift needle as illustrated in Fig. 4.

(A) Navigate to the “milling position” of the 100/400 mesh grid and adjust the stage to the

grid milling orientation (180° relative rotations to the loading angle and a 7° stage tilt).

(B) Proceed to a grid bar located at the left corner of the grid in order to attach a copper block to the bottom of the EasyLift needle.

(C) Insert the EasyLift needle and mill a flat bottom using a regular cross-section pattern (width, 15–20 μm ; z-depth, 20 μm ; 30 kV; 4 nA). All milling parameters are defined for use with a Xenon plasma source.

(D) Maneuver the EasyLift needle to the top of the grid bar until both surfaces make tight contact.

(E) Attach the bottom of the EasyLift needle to the copper block through redeposition milling, using an array of regular cross-sections (width, 0.5 μm ; height, 2.5 μm ; z-depth, 8 μm ; lateral spacing, 0.25 μm ; single pass; 30 kV; 0.3 nA).

(F) Release the block by milling the left and right sides of the copper grid bars using two line patterns (30 kV, 15 nA).

(G) Move the EasyLift needle to a higher position and retract the needle.

3 Trench milling of a sample block around the VOI position (Fig. 5 and Fig. 6).

(A) Navigate to the “milling position” of the sample carrier, move the VOI to the center of the SEM

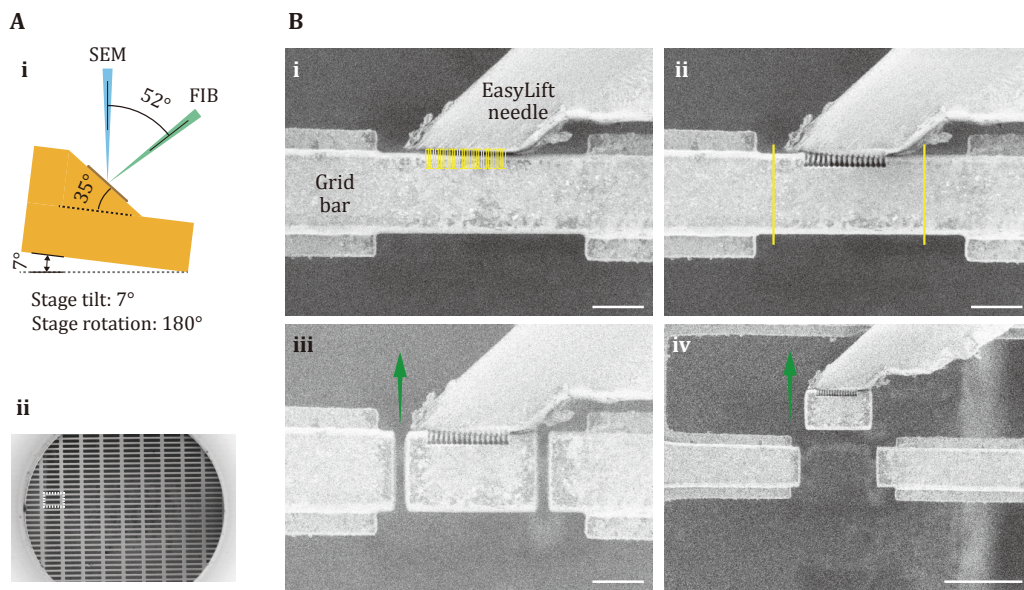


Fig. 4 Attachment of a copper block to the EasyLift needle. **A** Operational regions. (i) stage dimension and (ii) grid area for conducting the procedure, scale bars = 10 μm . **B** Procedure for attaching a copper block to the EasyLift needle. (i) Attach the flat bottom of the EasyLift needle to the pre-milled grid bar using an array of regularly spaced cross-sectional patterns, scale bar = 10 μm ; (ii) Mill through the grid bar using two parallel line patterns, scale bar = 10 μm ; (iii) Elevate the needle along with the copper block, scale bar = 10 μm ; (iv) Retract the needle while firmly securing the copper block, scale bar = 30 μm

image, and save the position as “Sample milling position”.

(B) Set the stage to the trench milling orientation (180° relative rotations to the loading angle and 7° stage tilt) and align the VOI position to be eucentric in both the SEM and ion beam images.

(C) Acquire a high-resolution ion beam image of the VOI using an appropriate magnification (exposure, 2 μs ; image size, 6144 $\times$ 4096; beam current, 13 pA).

(D) Mark the position of the sample block around the VOI using a rectangle measurement pattern as shown in Fig. 2.

(E) Release three sides of the sample block, as illustrated in Fig. 5A.

i. Mill an upper trench approximately 100 μm wide, 30–40 μm high, and 10 μm z-deep (30 kV, 60 nA), allowing sufficient space to maneuver the needle.

ii. Polish the bottom side of the trench using a lower current of 15 nA and a smaller pattern with a height of 2 μm .

iii. Mill the left and bottom trenches for the sample block at 15 nA using patterns of 65 $\mu\text{m} \times 5 \mu\text{m} \times 10 \mu\text{m}$, and 5 $\mu\text{m} \times 50 \mu\text{m} \times 10 \mu\text{m}$, respectively.

iv. Mill a portion of the right side to mark the edge at 15 nA using a pattern of 5 $\mu\text{m} \times$

20 $\mu\text{m} \times 10 \mu\text{m}$.

(F) Flatten the top surface of the sample block (Fig. 5B).

i. Rotate the stage by a relative 180° (stage tilt, 15°) to the sample top view orientation.

ii. Apply a new layer of GIS for 60 s to protect the freshly exposed front surface of the sample block.

iii. Mill the top surface using a rectangle pattern at 4 nA to produce a flat surface.

iv. Capture an SEM image to verify a flat and clean top surface for attaching the copper block to the EasyLift needle.

(G) Attach the EasyLift needle to the top of the sample block (Fig. 6A).

i. Insert the EasyLift needle after focusing the SEM image.

ii. Maneuver the EasyLift needle to the top center of the sample block until both surfaces make tight contact.

iii. Attach the copper block on the EasyLift needle to the sample block via redeposition milling, using an array of regular cross-sections on the copper block (width, 0.5 μm ; height, 3 μm ; z-depth, 5 μm ; lateral spacing, 0.5 μm ; single pass; 30 kV; 0.3 nA).

4 Lift out the sample block (Fig. 6B).

(A) Mill a line pattern across the lower part of the

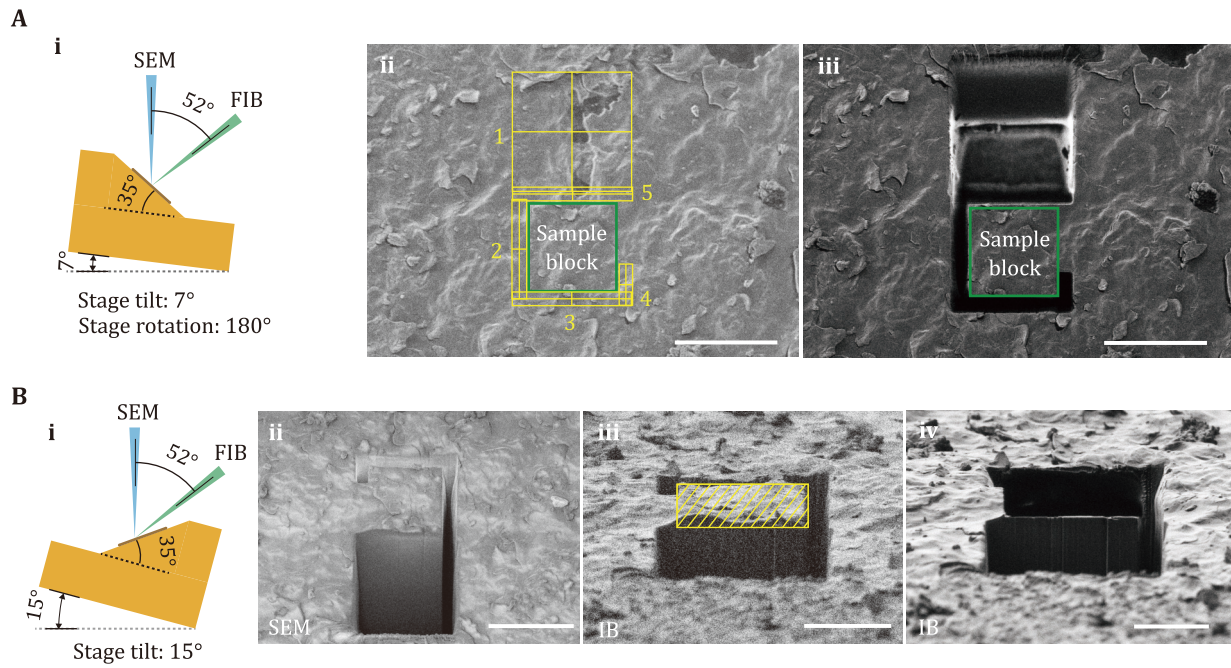


Fig. 5 Trench milling of a sample block. **A** Three-sided trench milling of a sample block surrounding the VOI position. (i) Illustration of the stage position; (ii) Pattern settings (labeled 1–5, indicating a regular cross-section pattern) used for trench milling to release the sample block (highlighted with a green rectangle); (iii) The IB image after trench milling. Scale bars = 50 μm . **B** Preparation of a flat surface for EasyLift needle attachment. (i) Illustration of the stage position; (ii) SEM image before milling; (iii) Pattern settings; (iv) IB image after milling. Scale bars = 50 μm (ii), 30 μm (iii), and 25 μm (iv), respectively

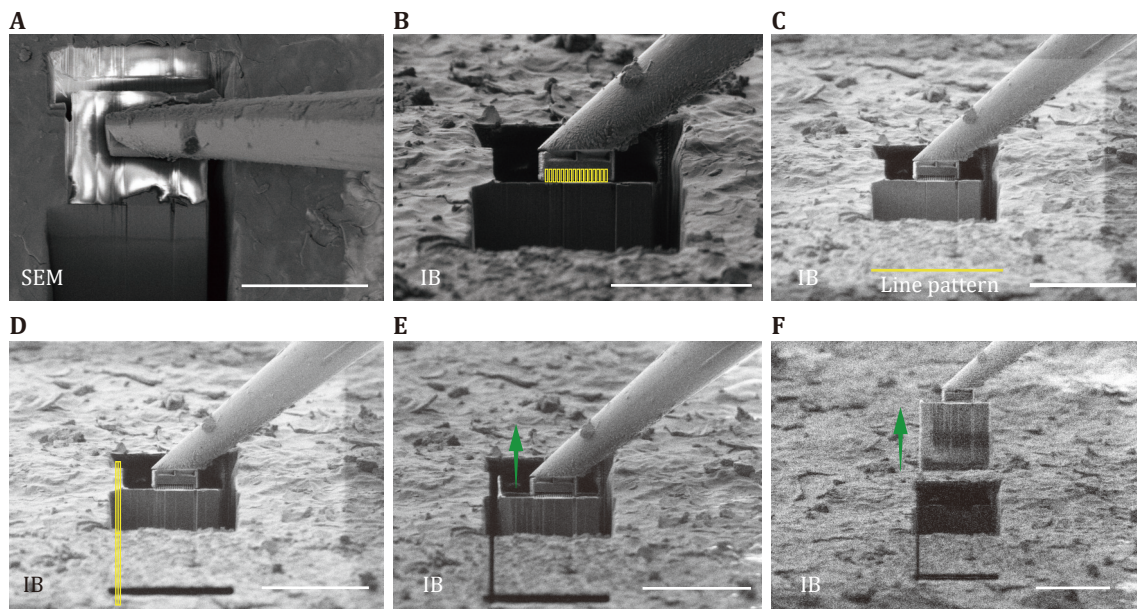


Fig. 6 Sample block lift-out procedure. **A** SEM image illustrating the position of the copper block on the top surface of the sample block. **B** IB image showing redeposition patterns on the bottom surface of the copper block. **C–E** Trench milling is performed using a line pattern (**C**) and an RCS pattern (**D**) to isolate the sample block. **F** The sample block is lifted out for subsequent processing. Scale bars = 40 μm (**A** and **B**), 50 μm (**C–F**)

sample block at a distance of 60 μm from the top of the block, effectively releasing it from the bulk material (15 nA; z-depth, 10 μm).

- (B) Mill the left side of the sample block (which corresponds to the right side in Step F (iv)) using a regular cross-section pattern of 5 $\mu\text{m} \times 60 \mu\text{m} \times 10 \mu\text{m}$ at 15 nA.
- (C) Move the needle in a stepwise fashion (step size, 200–500 nm) along the z-direction and verify that the sample block is fully released.
- (D) Elevate the needle with the sample block above the carrier bulk at low magnifications (650 $\times$ –100 $\times$).

(E) Retract the needle with the sample block.

Serial lift out of lamellae

- 1 Trim the sample block (Fig. 7Ai–7Aii).
 - (A) Position the system at the designated “Receiver position” as outlined in Step 2F of the “Sample Block Lift-Out” section.
 - (B) Navigate to the leftmost grid trenches.
 - (C) Center the two plateaus and adjust the focus.
 - (D) Insert the needle carrying the sample block and position it approximately 10 μm above the plateaus.

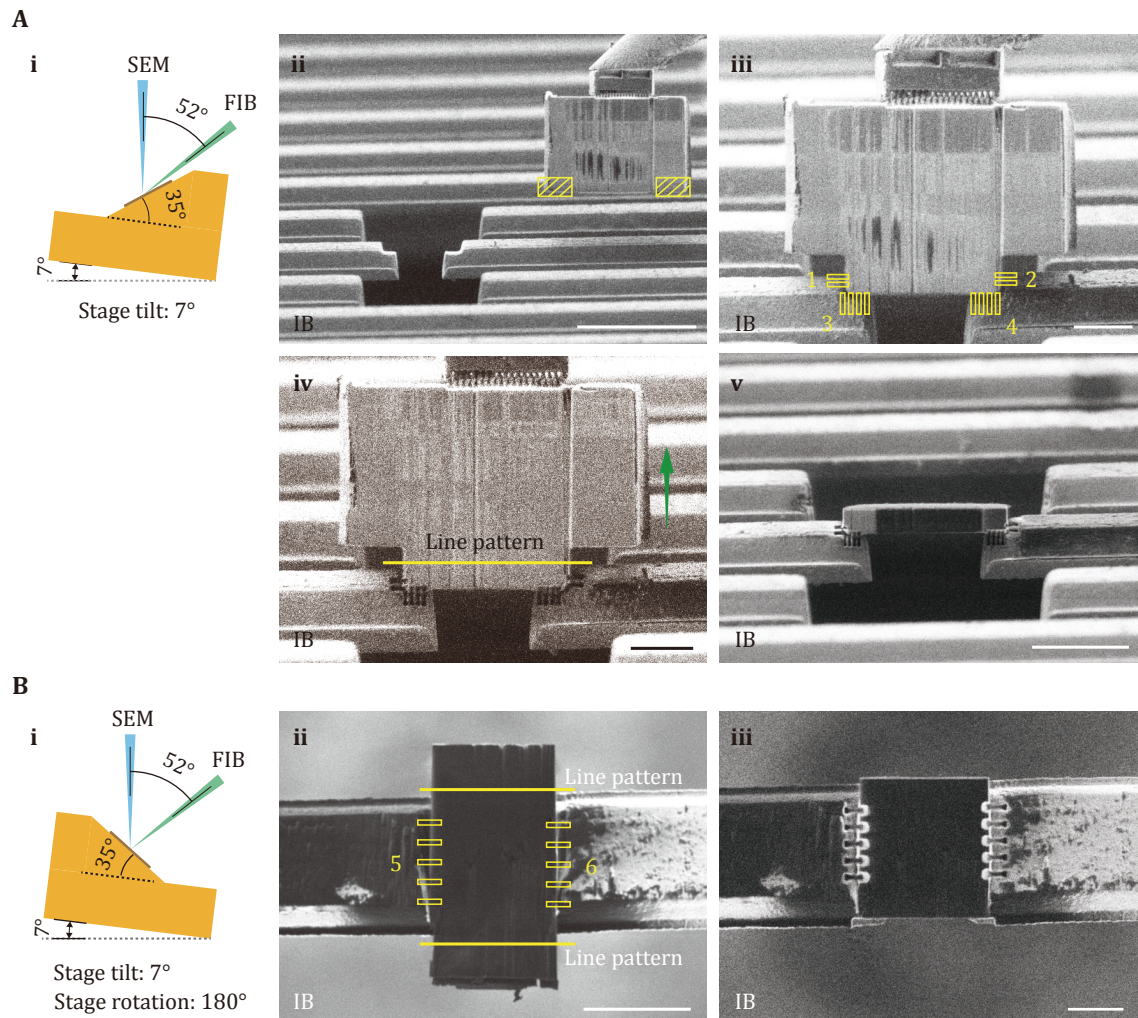


Fig. 7 Serial Lift-Out procedure of a lamella. **A** Generation and release of a lamella. (i) Illustration of the stage position; (ii) Trimming of the sample block, scale bar = 40 μm ; (iii) Attachment of the sample block on four sides using arrays of patterns, including the left and right sides (marked with pattern arrays 1 and 2) and the bottom left and right sides (marked with pattern arrays 3 and 4), scale bar = 10 μm ; (iv) Release of the first lamella from the sample block by milling a line pattern, scale bar = 20 μm ; (v) IB image of the released lamella, scale bar = 20 μm . **B** Attachment of the sample block to the top two sides of the lamella using two arrays of patterns (i–ii, marked with pattern arrays 5 and 6), followed by trimming of the front and back sides using two line patterns (ii–iii). Scale bars = 20 μm (ii) and 10 μm (iii)

- (E) Mill both sides of the sample block using two rectangular patterns (7 μm in height) at 4 nA to match the spacing between the two plateaus on the receiver grid.
- 2 Lower the sample block by manipulating the needle along the z-axis, and verify whether the sample block can be properly inserted into the two plateaus. If not, slightly raise the needle and repeat Step 1E.
- 3 Maneuver the needle to ensure the sample block makes firm contact with both plateaus.
- 4 Attach the sample block on four sides (Fig. 7Aiii).
 - (A) Mill the bottom left and right sides of the sample block using two arrays of regular cross-sections on the grid bar (width, 0.5 μm ; height, 3 μm ; z-depth, 5 μm ; lateral spacing, 0.25 μm ; single pass; 30 kV; 0.3 nA), respectively.
 - (B) Similarly, mill the left and right sides of the sample block using two arrays of regular cross-sections on the grid bar (width, 0.5 μm ; height, 3 μm ; z-depth, 5 μm ; horizontal spacing, 0.25 μm ; single pass; 30 kV; 0.3 nA), respectively.
- 5 Release the first lamella from the sample block by milling a line pattern (4 nA; z-depth: 5–10 μm) located approximately 4 μm above the lower edge of the sample block (Fig. 7Aiv).
- 6 Save the current stage position as “Lamella_01”.
- 7 Attach the sample block to the top two sides of the lamella (Fig. 7Bi–7Bii).
 - (A) Rotate the stage by 180° relative to the lamella's top view orientation (stage tilt, 7°).
 - (B) Mill the left and right sides of the sample block on the grid bar using an array of regular cross-sections on the grid bar (width, 3 μm ; height, 0.5 μm ; z-depth, 5 μm ; vertical spacing, 0.25 μm ; single pass; 30 kV; 0.3 nA).
 - (C) Retract the needle and sample block after slightly raising their positions.
- 8 Generate additional lamellae.
 - (A) Navigate to the second set of grid trenches at the previously defined “Receiver position” (refer to Step 2F of the “Sample Block Lift-Out” section).
 - (B) Reinsert the needle and transfer the sample block to rest atop the two plateaus.
 - (C) Repeat Steps 1–7 to produce the desired number of lamellae.
 - (D) In this instance, three lamellae were generated and labeled with the positions “Lamella_01”, “Lamella_02”, and “Lamella_03”.
- 9 (Optional) Trim the front and back surfaces of the lamellae using line patterns across the width of each lamella at 15 nA to remove excess sample block material extending beyond the 30- μm grid bar, as illustrated in Fig. 7Bii–7Biii.

Fine milling and polishing of lamellae

- 1 Go to top view orientation of lamellae and polish the front surface using a rectangle pattern at 1 nA. This step aims to produce a clean, flat surface while minimizing the occurrence of curtaining artifacts.
- 2 Apply a new layer of GIS for 60 s onto the freshly exposed front surface at the deposition position.
- 3 Rotate the sample stage by 180° relative to the defined lamella milling orientation (corresponding to a stage tilt of 7°) to reach the “Receiver position” (Fig. 8A).
- 4 Introduce a tension-release notch on one side of each lamella using fine milling at a beam current of 0.1 nA, following the procedure described by Pöge *et al.* (Pöge *et al.* 2025). The regular cross-section patterns are illustrated in Fig. 8B.
- 5 Perform progressive fine milling to achieve a thickness of 1 μm (Fig. 8C).
 - (A) Reduce the lamella thickness to approximately 2 μm by applying two rectangular milling patterns at the top and bottom regions of the lamella at 1 nA.
 - (B) Further thin the lamella to approximately 1.5 μm using rectangular patterns at a reduced beam current of 0.5 nA.
 - (C) Continue milling to a thickness of 1 μm using rectangular milling patterns at 0.3 nA.
 - (D) Proceed to the next lamella and repeat Steps 5A–5C until all lamellae are reduced to the target thickness of 1 μm .
- 6 Conduct final polishing to achieve a lamella thickness of approximately 200 nm (Fig. 8D).
 - (A) Navigate to the first lamella position, labeled “Lamella_01”.
 - (B) Mill down to a thickness of 0.5 μm using rectangle patterns at 50 pA.
 - (C) Further reduce the thickness to approximately 0.2 μm using rectangle patterns at 10 pA.
 - (D) Over-tilt the stage by 0.5–1.5° and mill the back of the lamella using a rectangle pattern at 10 pA.
 - (E) Move to the subsequent lamella and repeat Steps 6A–6D to complete the polishing process for all lamellae.

Sample retrieval

- 1 Unload the shuttle from the cryo-FIB microscope.
- 2 Transfer the sample carrier and TEM grid containing the lamellae into individual cryo-grid boxes, and store them in the liquid nitrogen until further use.

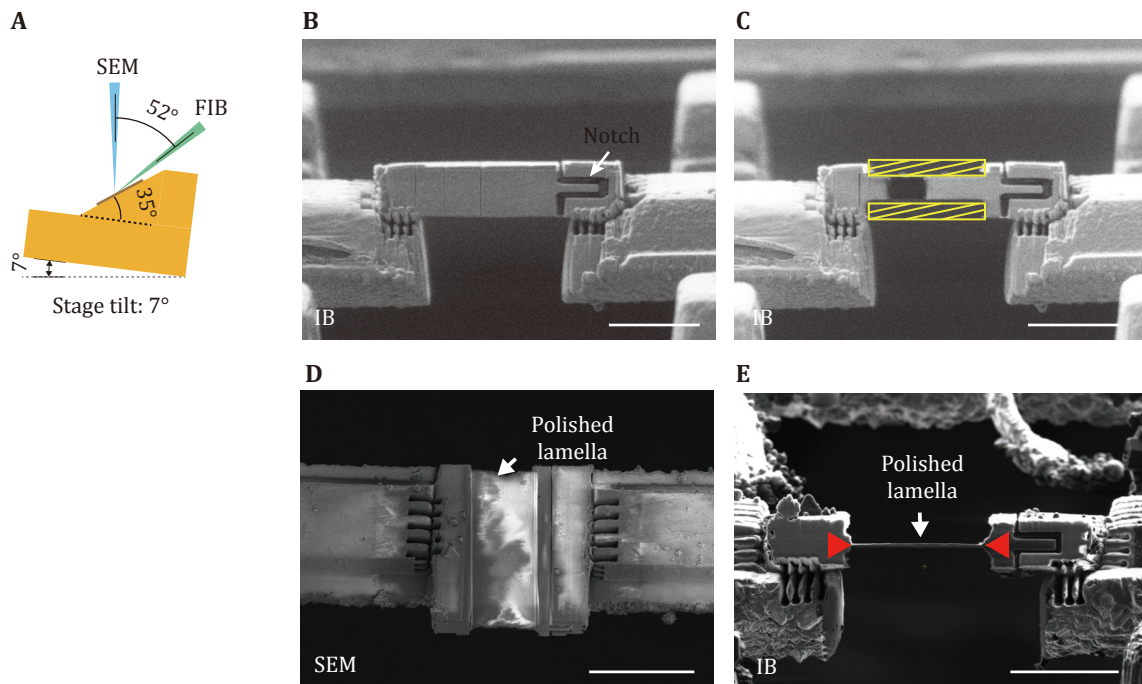


Fig. 8 Procedure for fine milling and polishing. **A** Illustration of the stage position. **B** Notch milling to relieve mechanical stress. **C** Reduction of lamella thickness to approximately 2 μm . **D-E** SEM and IB images of the final lamella, with a thickness of approximately 200 nm. White arrows indicate the position of the polished lamella; red arrowheads denote the width of the lamella. Scale bars = 20 μm (**B-D**) and 10 μm (**E**)

ANTICIPATED RESULTS

This protocol outlines a cryo-ET workflow tailored for mouse intestinal tissue samples, incorporating high-pressure freezing, CLEM, and an optimized Serial Lift-Out cryo-FIB milling procedure. Using this workflow, we successfully fabricated three vitrified, thin lamellae suitable for high-resolution tomographic imaging (Figs. 9A and 9B). An example of the resulting tomographic data is presented in Fig. 9C, which shows intestinal microvilli. The tomogram reveals clear delineation of the microvillar membrane, actin filaments within the core, and fine nanobristle-like structures decorating the membrane surface (Fig. 9C).

MATERIALS

Reagents

- Ficoll 400 (Sigma-Aldrich, Cat. No. F4375)
- Phosphate-buffered saline (PBS, Sigma-Aldrich, Cat. No. 10010023)
- L- α -Lecithin (Sigma-Aldrich, Cat. No. 429415-100GM)
- Reagent setup

- 40% Ficoll 400, dissolve Ficoll 400 in PBS (w/v), centrifuge or sonicate to remove air bubbles. Store at 4°C.

Equipment

- Helios™ 5 Hydra CX DualBeam plasma cryo-SEM/FIB system (Thermo Scientific)
- Leica EM ICE High-pressure freezer (Leica Microsystems)
- Type B sample carriers, 3 mm, 300 μm cavity, gold-coated copper (Leica Microsystems)
- Sapphire disc, 3 mm (Leica Microsystems)
- Spacer, 3 mm (Leica Microsystems)
- Grids, 100/400 mesh (Gilder Grids)

Software

- MAPS (Thermo Scientific)
- iFLM (Thermo Scientific)
- ImageJ software (Schneider *et al.* 2012)

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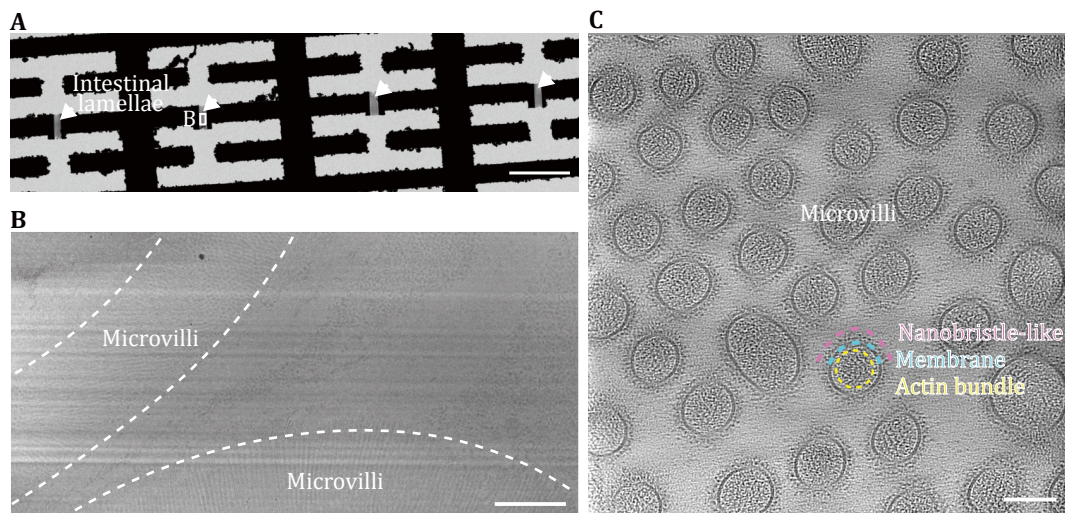


Fig. 9 TEM analysis of lamellae. **A** Overview TEM image of four lamellae fabricated using the described workflow. White arrows indicate the position of the polished lamellae, scale bar = 20 μ m. **B** Enlarged view of a lamella stack reveals distinct intestinal microvilli structures on the lamellae, scale bar = 20 μ m. **C** A representative tomographic slice of mouse intestinal microvilli, displaying a uniform distribution, with actin bundles (in yellow) located in the core and protein densities (in purple) observed on the membrane surface (in light blue), scale bar = 100 nm

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Author contributions M.L. designed research; X.T. performed research; All the listed authors analyzed data; and M.L. wrote the paper.

Compliance with Ethical Standards

Conflict of interest Xiaoyu Tang, Jiaming Liu, Jingtao Zheng, Huanhuan Huang, Zihang Yu, Ying Liu, Peng Wang and Meijing Li declare that they have no Conflict of interests.

Human and animal rights and informed consent All animal experiments were approved and conducted according to protocols approved by the Animal Experiments Committee at the Shenzhen Medical Academy of Research and Translation. All institutional and national guidelines for the care and use of laboratory animals were followed.

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