

# Making plant tissue accessible for cryo-electron tomography

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## Abstract

Cryo-Electron Tomography (cryo-ET) allows to visualize the molecular architecture of pristinely preserved cells and tissues. The workflow of sample preparation for cryo-ET is rather complex; it involves vitrification by rapid freezing followed by cryo-Focused Ion Beam (FIB) milling rendering the volumes of interest thin enough for cryo-ET data acquisition. The established protocols for single cells grown on or deposited on EM-grids are not suitable for multicellular plant tissues. Plunge-freezing does not yield vitrified samples in most cases and must be replaced by high-pressure freezing. This, in turn, necessitates extensive modifications of the subsequent FIB milling procedures. In this communication we describe procedures for sample screening, targeted FIB milling guided by cryo-fluorescence microscopy and a novel lamella trimming step that allows to obtain homogeneously thin lamellae suitable for cryo-ET. We have tested all the steps along the workflow with a variety of plant tissues including the moss *Physcomitrium patens* and tissues of *Arabidopsis thaliana* and *Limonium bicolor*. We could demonstrate that the workflow optimized for plant tissues allows to attain subnanometer resolution in cases where subtomogram averaging is applicable.

# Main text

New imaging modalities in conjunction with advances in sample preparation can pave the way for unprecedented insights into the structural organization of cells and tissues. Cryo-Electron Tomography (cryo-ET), i.e. the application of tomographic principles of image acquisition and reconstruction to biological materials in a frozen-hydrated state, allows to visualize the molecular architecture of unperturbed cellular environments<sup>1</sup>. It combines the power of high-resolution three-dimensional (3D) imaging with close-to-life structural preservation and provides faithful representations of the physiological state of cells<sup>2</sup>.

Instead of disrupting cells, fractioning their molecular inventory, and studying its components in isolation, cryo-ET allows to visualize them *in situ* embedded in their functional environments. Thereby, the network of interactions underlying cellular physiology is preserved and can be revealed<sup>3</sup>. Moreover, many supramolecular assemblies cannot be isolated without compromising their structural integrity. Advances in technology and methodology have established structural biology in cells or *in situ* as a viable alternative<sup>4</sup>. Today, cryo-ET is widely deployed and increasingly popular at the interface of molecular biology and cell biology – with exception of the plant sciences, where technical issues have hampered its use.

The critical part of the process is the vitrification of plant tissues by rapid freezing. Typically, plant cells are larger (diameter 10 – 100  $\mu\text{m}$ ) than yeast, algae or mammalian cells and they often contain solute depleted vacuoles occupying up to 90 % of their cellular volume<sup>5</sup>. Plunge-freezing<sup>6</sup>, the most convenient way to achieve vitrification, works reliably only for cells smaller than 10  $\mu\text{m}$ . While cryoprotectants<sup>7</sup> may be used to increase vitrification depth, for larger cells and tissues, High-Pressure Freezing<sup>8</sup> (HPF) must be applied and still not always guarantees full vitrification. Moreover, it is challenging to make HPF compatible with the subsequent step of the cryo-ET workflow: thinning the sample to make it suitable for electron tomography data acquisition. This

is achieved by Focused Ion-Beam (FIB) milling, where material is ablated from the ice-embedded sample until thin sheets (lamellae) with thicknesses of 100 - 300 nm are obtained<sup>9</sup>. To ensure that features of interest are included in the lamella, FIB milling is often guided by cryo-Fluorescence Light Microscopy (FLM), pinpointing the spatial location of structures of interest<sup>10,11</sup>. Recently, progress has been made in the preparation lamellae from HPF samples for cryo-ET<sup>12–20</sup>. These new procedures, however, have not yet been put into practice for plant tissue.

Therefore, almost all structural studies of plant systems have opted for an alternative route, namely HPF followed by freeze substitution. In this process, high-pressure frozen samples are dehydrated, metal-impregnated and embedded in a resin allowing sectioning and imaging at room temperature<sup>21</sup>. While this approach generally preserves cellular ultrastructure better than chemical fixation followed by metal staining and resin embedding<sup>22</sup>, it is impossible to ascertain that vitrification has been adequately achieved in the first instance. To visualize and interpret structures at the molecular level, the only suitable method is cryo-ET, with samples unadulterated by dehydration and chemical modifications.

In this study, we describe workflows suitable for the preparation of plant tissues for cryo-ET. First, we applied them to protonemata and phyllids of the moss *Physcomitrium patens*<sup>23</sup> forming one-dimensional chains and two-dimensional sheets, respectively, of cylindrical cells with diameters of 15-30  $\mu\text{m}$ . We employed FIB-milling guided by FLM, specifically targeting cell junctions, to show that features small compared to the size of whole cells can be addressed. Later we adapted the methods to several other species including more challenging tissues to demonstrate the potential of the workflows, which we believe are not limited to plants but widely applicable also to other, multicellular systems.

# Results

## Plunge-freezing does not vitrify *Physcomitrium patens* tissues

First, we applied the standard plunge-freezing and FIB milling workflow to *P. patens* protonemata and phyllids. As the cells were exposed on the surface of grids, the identification of cell junctions was possible using secondary electron images derived with the Scanning Electron Microscope (SEM) and FIB, without the need for fluorescence targeting by correlation to FLM data (Extended Data Fig. 1). Overviews of lamellae recorded in a Transmission Electron Microscope (TEM), however, revealed that the cells were not vitreous (Fig. 1a, N = 15). The formation of large ice crystals during plunge-freezing resulted in distortion of the cellular architecture and the generation of severe TEM imaging artefacts.

We also performed plunge-freezing with glycerol, sucrose and proline additives serving as cryoprotectants (Table 1). These, however, did either not prevent crystalline ice formation or induce plasmolysis (data not shown). In some cases, the tissue was even severely dehydrated (Extended Data Fig. 1f). Thus, we concluded that it is challenging to vitrify *P. patens* tissues by plunge-freezing. While the success of plunge-freezing is sample dependent, we anticipate similar vitrification problems for vascular plants and mosses with cells of similar or larger size.

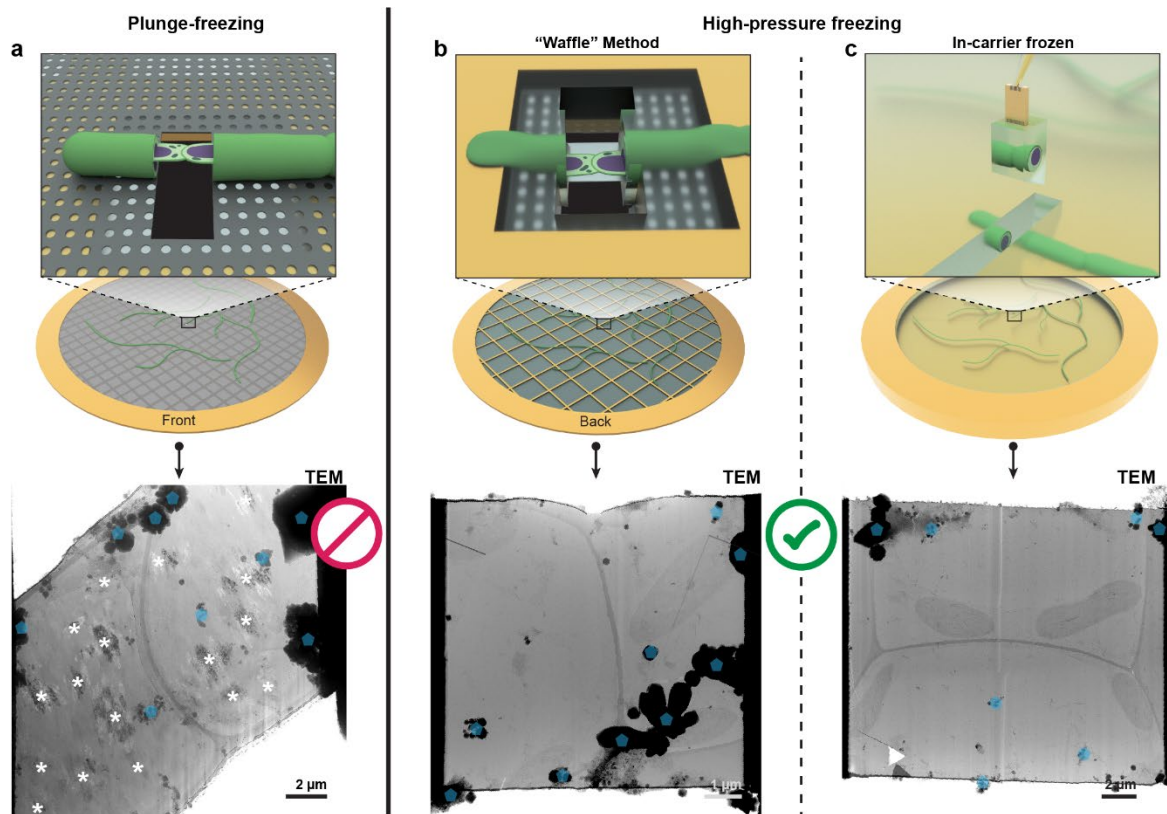
## The “Waffle” Method utilized for *P. patens*

Next, we tested the “Waffle” Method<sup>18</sup> to vitrify protonemata. The tissue was placed on the back of an EM-grid, i.e. on the reverse side of a continuous support film and squeezed into the buffer-filled wells formed by the EM-grid bars and support film prior to HPF. While this approach damages cells on top of grid bars, the cells within grid squares remain intact. Hence, using EM-grids with large squares (50-100 mesh) maximizes the area of intact, vitreous tissue.

In contrast to plunge-frozen samples, the cells on “waffle” grids are embedded in a layer of vitreous buffer and not exposed at the sample surface (Extended Data Fig. 3a). Therefore, we screened “waffle” grids in a cryo-FLM recording the green cellular autofluorescence to identify single layers of intact protonemata tissue with cell junctions in the center of grid squares (Fig. 2a,b). Furthermore, squares with multiple cell layers were ignored as only a single layer of protonemata fits into the 25  $\mu\text{m}$  high well of the used grids without mechanical damage caused by compression (Extended Data Fig. 3b-c).

Subsequently, we loaded the samples into a FIB/SEM instrument equipped with an integrated Fluorescence Light Microscope (int-FLM) for lamella preparation. While lamella milling of plunge frozen samples can be done by ablating material with the FIB from a shallow angle, milling “waffle” grids requires additional steps. First, the target area must be located laterally, i.e. parallel to the sample surface. Then holes, referred to as trenches, are milled around the target area with the FIB axis perpendicular to the sample surface (trench milling orientation, Extended Data Fig. 2). Afterwards, the axial position of the target area is determined followed by thinning the sample from a shallow angle to obtain a lamella (lamella milling orientation, Extended Data Fig. 2).

Correlating the FLM images with FIB images is difficult due to the near-featureless surface of “waffle” grids. For lateral targeting, a marker hole was milled in trench milling orientation (Fig. 2c) into a grid square containing a target area identified previously in the FLM. Marker and target area were then localized in sample top views recorded with the int-FLM (Extended Data Fig. 2d) and the measured distance between them was used to guide trench milling (Extended Data Fig. 2e).

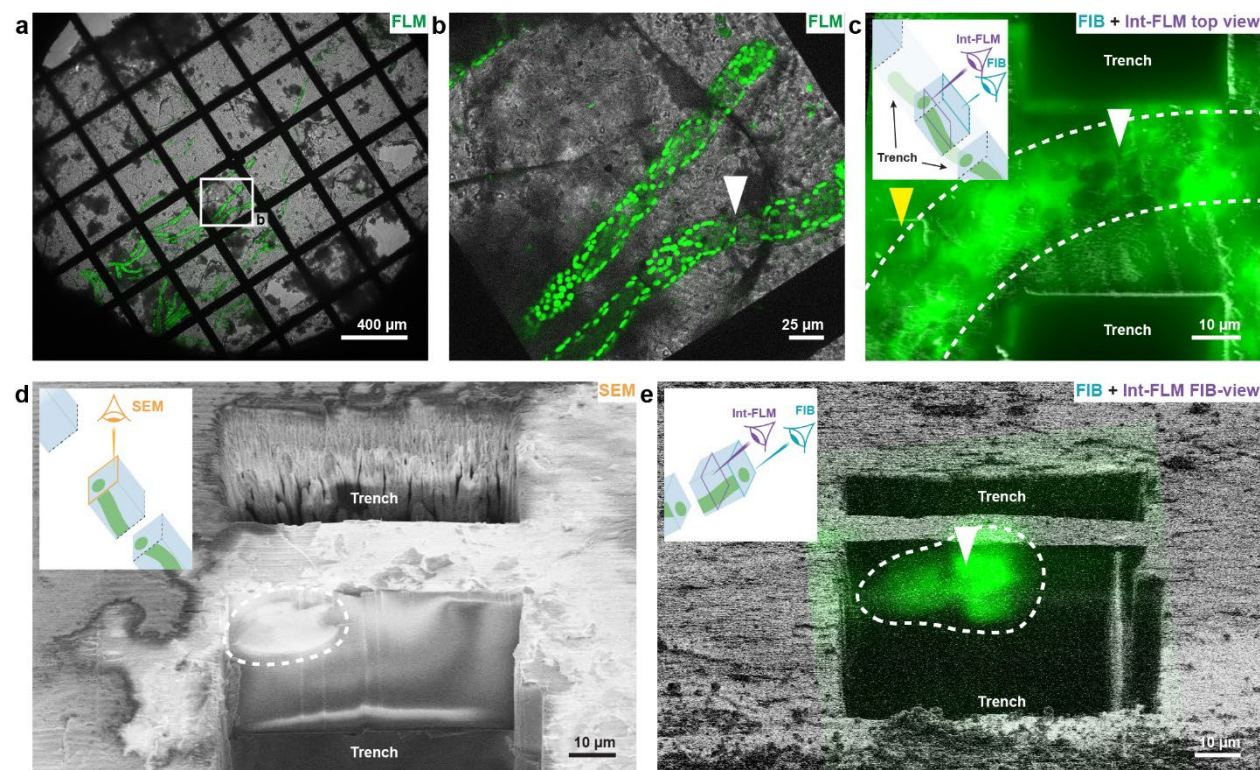


**Fig. 1 | Plunge-freezing versus High-Pressure Freezing (HPF) for the moss *Physcomitrium patens*.** The top and bottom row show schemes of the samples and corresponding examples of TEM overview images, respectively. **a**, Plunge-freezing. Patterns introduced by crystalline ice are indicated by asterisks (\*). **b,c**, High-pressure freezing. Samples prepared by the "Waffle" Method or in-carrier HPF in (**b**) and (**c**), respectively. TEM overviews of lamellae from HPF samples show no indication of crystalline ice. In (a)-(c), ice crystals contaminating the lamella surface which were introduced during transfers between microscopes are marked by blue transparent pentagons.

To ensure that the cell junction is contained within the final lamella with a thickness of only a few hundred nanometers, it must also be targeted in the axial dimension, i.e. within the thickness of the sample. For plunge frozen samples, 3D-correlative light microscopy and FIB milling allows axial targeting by using cryo-FLM z-stacks<sup>10</sup>. This approach, however, is difficult to apply to thick samples, because of poor axial resolution, depth dependent aberrations and difficulties in registration between 3D-stacks and FIB images of HPF samples. We applied two different imaging modalities for axial targeting. First, cellular components like cell walls, membranes or organelles can be visualized by SEM block-face imaging<sup>24</sup>, if they are exposed at the FIB milled

surface after trench milling (Fig. 2d, Extended Data Fig. 2c). Second, when the target area is buried beyond the surface, the int-FLM can be used in the “FIB-view” mode (Capitanio *et al.*, will soon be described in a manuscript). Here, int-FLM images are not acquired as top views but with the stage in lamella milling orientation (Extended Data Fig. 2b) from the same shallow angle at which the lamella is thinned to its final thickness (Fig. 2e).

Next, the lamella was further thinned in several successive steps (Extended Data Fig. 4). At a thickness of 6  $\mu\text{m}$  a notch similar to reference<sup>18</sup> but larger was milled to allow for movements of more than 1  $\mu\text{m}$  to prevent lamella bending in the type of samples studied here (Extended Data Fig. 4b). Afterwards, the lamella was thinned to its final thickness of 100-300 nm (Extended Data Fig. 4c) and imaged in a TEM. Lamella overviews reveal the subcellular organization in *P. Patens* protonemata unperturbed by crystalline ice (Fig. 1b, Fig. 3d).



**Fig. 2 | Sample screening and targeted lamella preparation.** a,b, Sample screening using a cryo-fluorescence light microscope (FLM). An overview of a “waffle” grid is shown in (a). The white rectangle indicates the position of the grid squares in (b) containing a target area. Displayed in (a) and (b) are the transmitted light channel (gray) and the cellular autofluorescence (green). c, Lateral

targeting. Overlay of a FIB image (gray) and cellular autofluorescence (green) recorded with the integrated Fluorescence Light Microscope (int-FLM). Both images (Extended Data Fig. 3g) were acquired with the stage in trench milling orientation resulting in sample top views. **d,e**, axial targeting. **d**, SEM image of the block-face exposed through trench milling. **e**, Overlay of FIB (gray) and int-FLM FIB-view image (green) of the cellular autofluorescence. Both images were recorded with the stage in lamella milling orientation. Cell junctions and locations of cells are marked with white arrowheads and dashed lines, respectively, in (**b-e**).

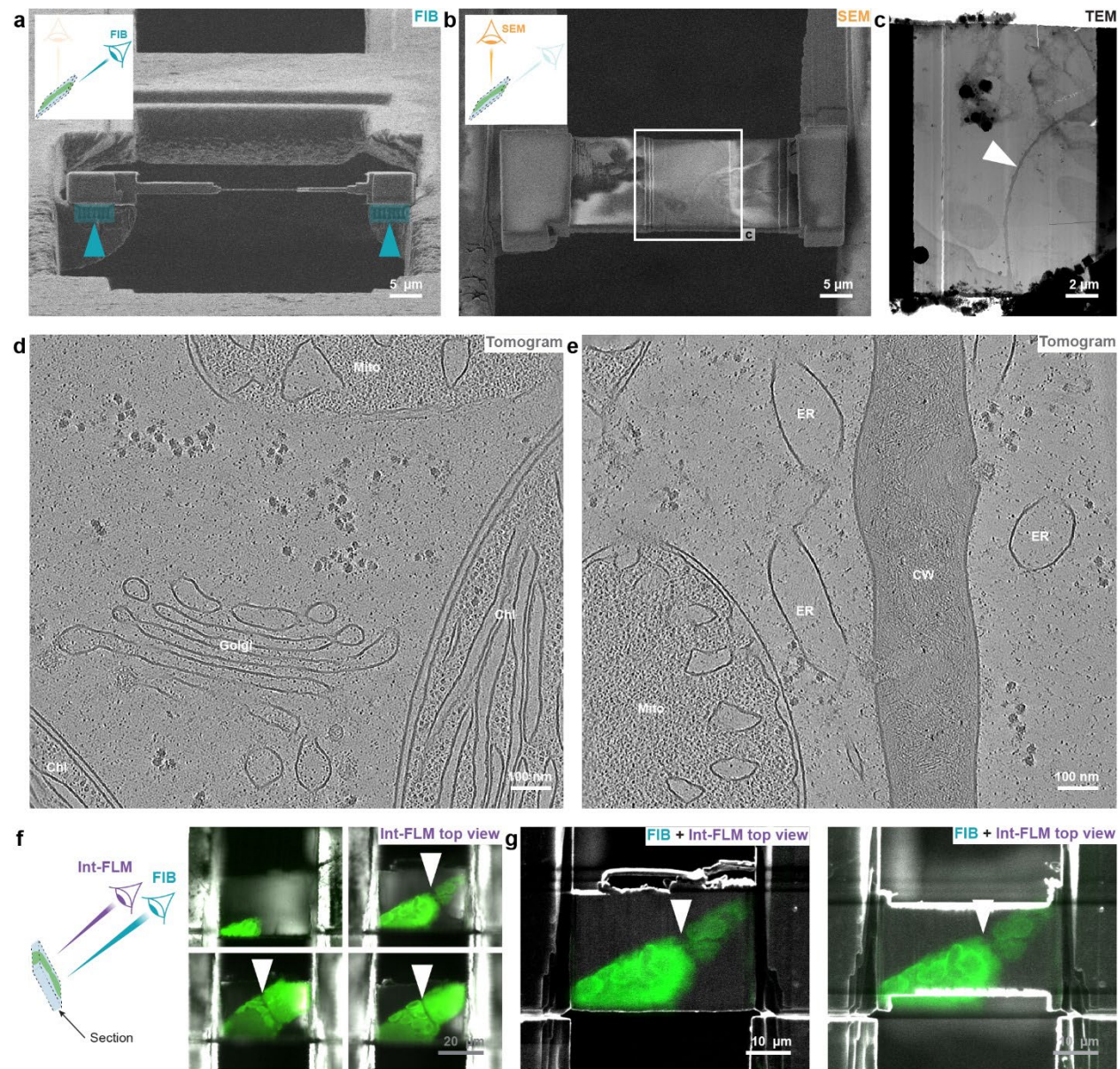
## Optimization of Serial Lift-Out for lamella quality

The cell junctions we targeted in this work have a diameter of 15-25  $\mu\text{m}$ . The process of “waffle” milling results in a single lamella of a few hundred nanometers in thickness. Thus, about 98% of the targeted volume is lost. Additionally, as targeting and trench milling are time-consuming processes, the yield of a single lamella per site renders the technique low throughput. An alternative to “waffle” milling is Serial Lift-Out<sup>19</sup>. Here, the entire target area is extracted by lift-out (Supplementary Movie 1 and 2) and cut into several sections that are attached to a receiver grid. Those sections are then fine milled, resulting in multiple lamellae sampling the target area, increasing the throughput and the contextual information obtained<sup>19</sup>.

We explored both attachment methods proposed in the original paper: single-sided and double-sided attachment. For the single sided attachment, redeposition milling was used to attach only one side of a section to a pin (Extended Data Fig. 6, Supplementary Movie 3). In our hands, this was the simplest attachment method as it required less precise maneuvering of the lift-out system, but the resulting lamellae often bent during fine milling (Extended Data Fig. 6f), especially when thinned below 200 nm as described previously<sup>19</sup>. Lamella loss during the transfer between microscopes was also more frequent (Supplementary Figure 6). Furthermore, lamellae were unstable during data acquisition, often resulting in poor data quality.

For the double-sided attachment, the width of the lift-out block was adjusted to fit precisely between two grid bars of a 400x100 mesh grid. Attachment of the section on both sides (Extended Data Fig. 7, Supplementary Movie 4) prevents bending during fine milling and results in lamellae

which are stable during microscope transfers and data acquisition. This approach, however, requires more precision with the micromanipulator system and user experience.



**Fig. 3 | Serial Lift-Out with double sided attachment from below and section trimming.** a-c, Double-sided attached lamellae with the attachment from below. FIB and SEM images are shown in (a) and (b), respectively. The attachment sites are marked in blue. The white rectangle in (b) marks the field of view in (c). c, TEM overview image of the lamella. d,e, Single slices of denoised tomograms. Chl indicates Chloroplasts, Mito Mitochondria, ER the cortical Endoplasmic Reticulum and CW the cell wall. f, Four Int-FLM top views of successive Serial Lift-Out sections (reflected light in gray and cellular autofluorescence in green). The junction

between cells (white arrowhead), is only contained in three of them. **g**, Overlay of FIB (gray) and int-FLM top view images (green) of the top right section in (f) before and after section trimming in the left and right panel, respectively.

In the context of the present study, we adapted the double-sided attachment method to focus on lamella quality rather than quantity. To this end, we milled plateaus into grid bars onto which the lift-out block could then be placed. The sections were then attached to the plateaus from below (Fig. 3a, Extended Data Fig. 8, Supplementary Movie 5). While milling the plateaus increases the expenditure of time (15-25 min per section), it leads to robust attachment and is easier to learn for lift-out beginners because it requires less precision concerning the dimensions of the lift-out block. The reduced precision requirement also allows Serial Lift-Out to be performed on FIB systems with stage instabilities or with imperfections in the FIB optics. For all three attachment methods, we obtained vitreous lamellae (Fig. 1b, Fig. 3c) and recorded tomograms revealing the subcellular organization of *P. patens* with an unprecedented level of detail (Fig. 3, Supplementary Movie 6).

Generating homogeneously thin lamellae is challenging, especially if they are larger than 10-20  $\mu\text{m}$  along the milling direction. To improve lamella quality, we included an additional step to the Serial Lift-Out workflow, namely section trimming. After the attachment, int-FLM top views were acquired for all sections to check which of them contained the target area (Fig. 3f). This also allowed to identify regions of the sections with no features of interest which were removed by FIB milling in trench milling orientation (Fig. 3g), minimizing the depth of the lamella in the thinning direction. During the lift-out process, the front of the lift-out block becomes damaged and uneven due to repeated ion exposures. At least 2  $\mu\text{m}$  of the section front were also removed to create a new, smooth front. This trimming step makes fine milling faster because less material must be ablated and allows to generate homogeneously thin lamellae as the smooth front prevents curtaining<sup>25</sup>.

## Lift-out in different stage orientations

On “waffle” grids of *P. patens*, the axis of the cylindrical protonemata cells is always roughly parallel to the grid surface. Thus, on-grid “waffle” milling and lift-out with the stage in the lamella milling orientation (Extended Data Fig. 5 left panel, Supplementary Movie 1) will inevitably yield sections roughly parallel to this axis (longitudinal sections). Performing lift-out in the trench milling orientation on cells with the protonemal axis oriented roughly parallel to the lift-out direction, cross-sections can be obtained (Extended Data Fig. 5 right panel, Supplementary Movie 2), analogous to *Caenorhabditis elegans* L1 larval cross-sections in reference<sup>19</sup>. Thus, it is possible to address samples which can only be frozen in distinct orientations from different directions, which is crucial to study subcellular structures with defined orientation within cells.

## In-carrier high-pressure freezing

During “waffle” freezing, *P. patens* protonemata can be damaged for two reasons. First, the filaments are larger than the squares of the EM-grids used and cells running across grid bars will be squeezed. Second, often multiple cell layers were pressed into the 25 µm high well formed by the grid bars which is not high enough to accommodate them without compression. To avoid this mechanical damage, we high-pressure froze protonemata directly in HPF carriers with a 2 mm wide and 100 µm deep cavity.

While the procedure of in-carrier high-pressure freezing is fast, it comes with additional challenges. First, samples cannot be screened easily in a FLM because carriers do not fit into the commercial shuttle. Thus, montage overviews were recorded with the int-FLM (Extended Data Fig. 9a). In our systems, this takes longer and yields poorer quality images due to widefield imaging and the low numerical aperture of the objective. Nevertheless, the obtained montage overviews were sufficient to identify target areas. Trench milling aided by int-FLMs was then performed as described in the previous section (Extended Data Fig. 9b).

Second, lamellae must be prepared by lift-out methodologies as trench milling through the carrier is impractical. Additionally, the lift-out block must be detached from the bulk also from below and not only on all four sides as for “waffle” grids. This requires milling an undercut after trench milling in lamella milling orientation (Extended Data Fig. 9c, Supplementary Movie 7 and 8).

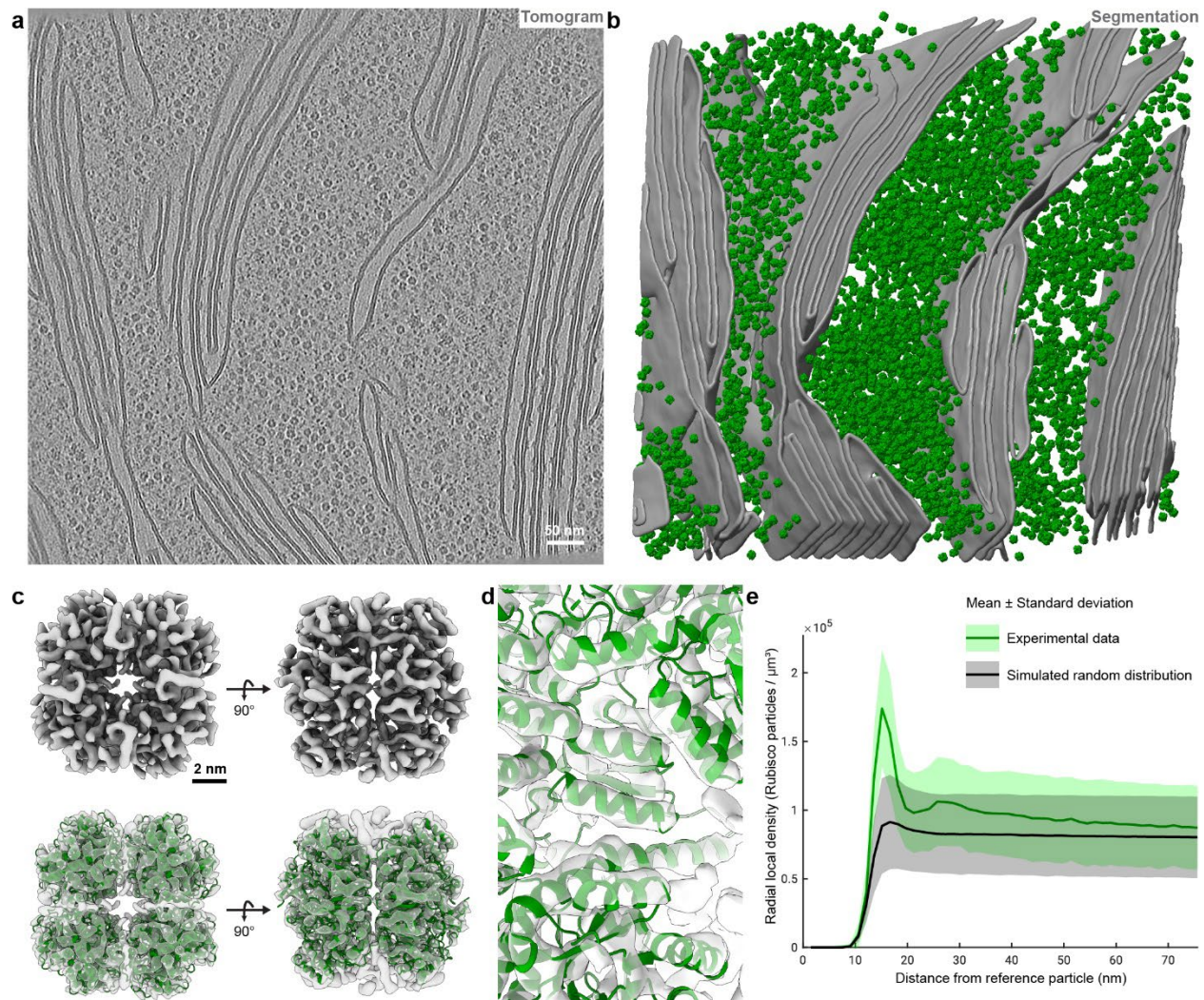
Third, in-carrier HPF samples are thicker than typical “waffle” grids. Thus, the axial targeting via SEM block-face imaging or FIB-view imaging with the int-FLM is crucial to ensure that the undercut is milled below the target area and that it is extracted completely (Extended Data Fig. 9c). Apart from these technical complications, lift-out and sectioning was then performed as for “waffle” samples, with the added benefit that in-carrier HPF potentially allows to vitrify thicker samples.

#### Subtomogram averaging of rubisco

In order to test if structural details are preserved throughout the presented workflows, we subjected rubisco<sup>26</sup>, an abundant enzyme in chloroplasts, to subtomogram averaging<sup>27</sup>. We acquired a small dataset composed of 10 tomograms on *P. patens* chloroplasts, picked rubisco positions by template matching and computationally aligned and classified subtomograms. Fig. 4a shows an example of a chloroplast tomogram (Supplementary Movie 9), and Fig. 4b the corresponding 3D rendered image of segmented chloroplast membranes with an intermediate, low-resolution rubisco average pasted at the determined particle positions (see supplementary material). We docked a model of an octamer of large rubisco subunits predicted by AlphaFold 3<sup>28</sup> into the final average of about 6000 subtomograms (Fig. 4c). The  $\alpha$ -helices of the predicted model fit well into the experimental density (Fig. 4d) indicating that we attained an average with subnanometer resolution (Supplementary Figure 4,  $FSC_{@0.143} = 0.79$  nm,  $FSC_{@0.5} = 0.95$  nm).

Based on the determined positions of rubisco complexes within tomograms, we calculated the local rubisco density which has a peak at 16 nm in contrast to a simulated random distribution

(Fig. 4e). This indicates that rubisco is not randomly distributed within *P. patens* chloroplasts similar to previous findings in the pyrenoid of the green alga *Chlamydomonas reinhardtii*<sup>29</sup>. These results demonstrate that subnanometer structural details are preserved throughout the presented workflows and that it is now possible to perform *in situ* structural biology studies in multicellular plants.



**Figure 4 | Subtomogram averaging of rubisco.** **a**, Slice of a denoised tomogram recorded on a chloroplast (Supplementary Movie 9). **b**, Three-dimensional rendered representation with segmented chloroplast membranes (gray) and the rubisco subtomogram average (green) pasted at the positions determined after template matching and subtomogram classification. **c**, Rubisco subtomogram average in isosurface representation (top row) and an octameric model of large rubisco subunits predicted by AlphaFold 3 docked into the average (bottom row). **d**, Close-up view of the average shown in (c) revealing that predicted  $\alpha$ -helices fit well into the experimental density map. **e**, The local density of rubisco particles as a function of the distance from the reference particle. Green line

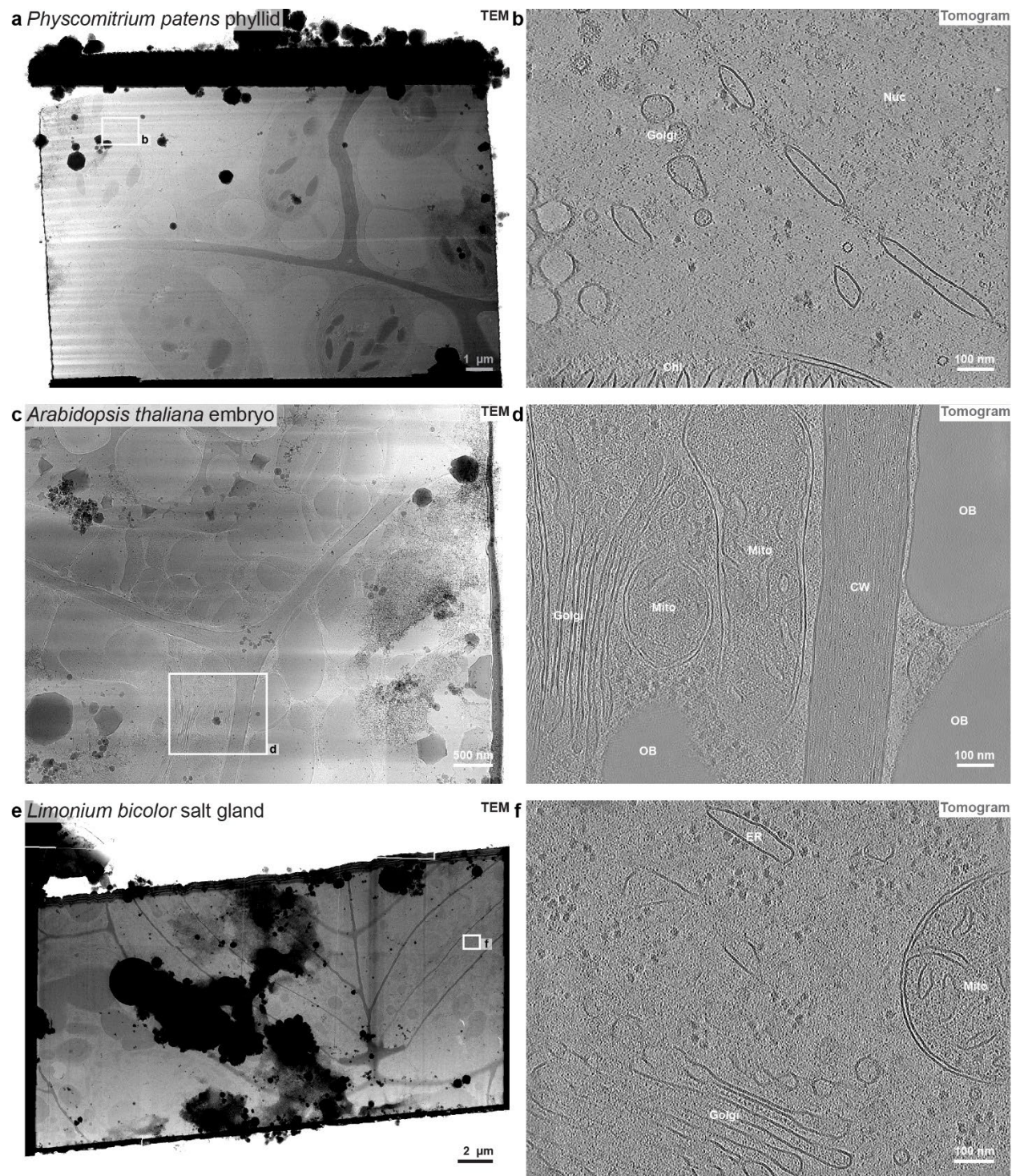
and area are the average of experimental density  $\pm$  one standard deviation, respectively, obtained from 10 tomograms. Black line and gray area are the mean  $\pm$  one standard deviation obtained for a simulated random particle distribution.

## The high-pressure freezing workflow applied to other tissues and plants

To demonstrate the application of the HPF methods presented to other tissues and plant species, we further used it on phyllids, the leaf-like structures formed by *P. patens*. Small pieces of phyllids were dissected and frozen with the “Waffle” Method. The samples were screened to avoid investigating areas which have been damaged during dissection or freezing (Extended Data Fig 10a-c). We were able to obtain vitreous lamellae and tomographic data from this tissue (Fig. 6a,b, Supplementary Movie 10).

Next, we adapted the “waffle” workflow for *Arabidopsis thaliana* embryos. While the embryos easily fit into grid squares, they are with about 50  $\mu$ m thicker than the 25  $\mu$ m high bars of EM-grids used here (Extended Data Fig. 10d,e). To avoid mechanical damage, an additional spacer ring was added onto the grid before “waffle” freezing, which increased the thickness of the “waffle” sample to around 75  $\mu$ m and prevented squeezing of the embryos in the process (Extended Data Fig. 10d,e). *A. thaliana* embryos were successfully vitrified, and tomographic data acquired (Figure 5c-d, Supplementary Movie 11).

As a last study subject, we high-pressure froze the first true leaves of *Limonium bicolor* (Extended Data Fig. 10f) in the 300  $\mu$ m deep cavity of HPF carriers. We targeted the blue autofluorescent salt glands (Extended Data Fig. 10g-h) again aided by the int-FLM and performed Serial Lift-Out. A TEM overview of a salt gland section and a tomographic slice are depicted in Fig. 5e and f, respectively, revealing its cellular architecture in a near-native state (Supplementary Movie 12). These results illustrate that the presented HPF methods are broadly applicable.



**Fig. 5 | The cryo-ET workflow applied to other tissues and species. a,b, *Physcomitrium patens* phyllid sample. a, TEM overview image of a single-sided attached lamella. b, a single slice of a tomogram recorded on the nuclear envelope (Supplementary Movie 10). c,d, *Arabidopsis thaliana* embryo sample. c, Low magnification TEM image of a lamella. d, Single tomographic slice of the cell wall between cells (Supplementary Movie 11). e,f Salt gland of *Limonium bicolor*. e, TEM overview image of a double-sided attached**

297 lamella. **f**, Single slice of a tomogram. Chl indicates Chloroplasts, Mito Mitochondria, OB oil bodies, Nuc the nucleus, CW the cell wall  
 298 and ER the Endoplasmatic reticulum. All tomographic slices are from computationally denoised tomograms (Supplementary Movie  
 299 12).

# Discussion

Compared to the “standard” cryo-ET workflow, comprising plunge-freezing, on-grid lamella milling and data acquisition, working with high-pressure frozen samples is technologically more demanding and has a lower throughput and success rate. Hence, if a sample can be vitrified by plunge-freezing, it still is the method of first choice. We must assume, however, that most plant tissues are too thick to be vitrified by plunge-freezing and must instead be high-pressure frozen.

Depending on the size of the sample, there are several high-pressure freezing variants. For the “Waffle” Method without spacer ring, the thickness of the EM-grid defines the maximum thickness of the sample. While standard EM-grids have a thickness of 20-25  $\mu\text{m}$ , thicknesses between 8-100  $\mu\text{m}$  are commercially available. Additionally, the shape (e.g. square, hexagon, hole and slot) and the size of the mesh can be chosen to match the shape of the tissue. For samples exceeding 100  $\mu\text{m}$  thickness, spacer rings can be added, as exemplified here on *A. thaliana* embryos. The advantage of “waffle” grids is that they allow on-grid lamella preparation and no undercut is required when performing lift-out. Samples with diameters of up to 2 mm and a maximum thickness of 200-300  $\mu\text{m}$  can potentially be high-pressure frozen in HPF carriers without risking squeezing damage by grid bars. In this case, on-carrier lamella preparation is currently technologically impractical and lift-out methodologies are required.

Vitrification is fundamental for preserving the sample in a near-native state and has a strong impact on the cryo-ET data quality and subsequently subtomogram averaging. We obtained data from clearly vitrified, thus apparently structurally intact samples in many cases. The success rate, however, was very sample-dependent. All our *A. thaliana* embryo and *L. bicolor* samples were vitreous (N = 4 and N = 1, respectively), perhaps due to the cells, which are densely packed with cellular components and the absence of large vacuoles. For *P. Patens*, the success rate was 47% (N = 45, Supplementary Figure 6a). Occasionally, even cells from within the same grid square

differed in their vitrification state. We applied similar methods to the trichomes of *Nicotiana benthamiana* and observed large ice crystals in all of them (N = 10). This illustrates that obtaining consistent vitrification results with high-pressure freezing can still be a major challenge for some samples. Moreover, certain samples require harvesting or dissection before being manually mounted in HPF carriers, which inevitably damages or stresses the cells and may result in artifacts. Hence, the preparation of samples for HPF should be as soft as possible while minimizing the perturbation of the system under investigation. It would therefore be beneficial to develop new protocols or even methods, that are largely user independent or completely automated, highly reproducible and capable of even vitrifying larger and thicker tissues<sup>30,31</sup>.

Additionally, HPF samples demand a targeted approach, since the cells are not exposed on the sample surface but submerged in a layer of vitreous buffer. The localization of target areas requires fluorescent markers or autofluorescence. For lateral targeting in the x-y-plane of the sample, grids were screened in a stand-alone cryo-FLM to identify suitable grid squares. Next, int-FLMs proved practical to guide trench milling. To ensure that the target area is contained within the final lamella or lift-out block, its axial position (location within sample thickness) must be determined. SEM block-face imaging after trench milling is one option. It is only applicable, however, if sufficiently contrast-rich features, like membranes, in the vicinity of the target area are exposed at the block-face. The second option is FIB-view imaging which can be applied if a fluorescently labelled or autofluorescent target is discernible in int-FLM images.

On-grid “waffle” milling has the lowest methodological entry barrier and does not require lift-out. It is faster for screening several locations per grid and assessing the sample quality than Serial Lift-Out. At the same time, it exhibits a lower throughput by obtaining only a single lamella per targeted area. Additionally, fine milling for our “waffle” samples must be done at relatively high stage tilts (20°) to fully access the target area and it is often difficult to remove all material below lamellae. Both factors restrict the angular range for tomographic data acquisition and, in turn, compromises data quality. For large target areas, like in our case the junction between cells,

Serial Lift-Out is the preferred method. While it requires some training and a lift-out system, after sectioning, fine milling is faster and more straightforward than for “waffle” milling and can be performed at shallow angles. The full  $\pm 60^\circ$  tilt range for cryo-ET, can therefore be accessed. For plant samples, single-sided attachment is the fastest lift-out approach requiring least user precision. But smaller lamella dimensions, potential bending and instabilities during data collection must be considered. In comparison, double-sided attachment yields lamellae that are easier to thin homogeneously without bending and produce higher quality data. For the double-sided attachment, we tested two methods. The side-attachment (i), as described originally in reference<sup>19</sup> needs little modifications of the receiver grid and consequently requires less expenditure of time. Yet, fitting the lift-out block precisely between the grid bars allows only for an error of a few hundred nanometers. Hence, it requires most user expertise, a mechanically very stable stage and lift-out system as well as well-aligned ion optics. The attachment from below (ii) is more time-consuming due to the additional generation of plateaus but adjusting the size of the lift-out block and placing it on the plateaus tolerates manipulation errors of a few micrometers. Therefore, the attachment from below is likely easier to adopt for lift-out novices. The additional trimming step introduced here focuses on obtaining highest lamella quality over quantity. First, int-FLM top views of Serial Lift-Out sections confirm that the target area is captured within followed by removal of regions that do not contain material of interest in trench milling orientation. This minimizes the lamella size in the fine milling direction and centers the target between smooth fronts which is important to obtain homogeneously thin lamellae during the subsequent fine milling in lamella milling orientation. To further increase the throughput and reduce the experience defined entry barrier, automation is important. Since we explored multiple samples which all behaved differently, all milling steps were performed manually. The whole lamella preparation workflow, however, is per-se very repetitive and time-consuming. Several software tools already exist<sup>15,32–34</sup> which can be used for

on-grid “waffle” milling and fine milling of lift-out sections. Yet, robust automated protocols for targeted trench milling, lift-out and sectioning must still be developed.

In summary, we applied various HPF and lamella preparation methods to different plant species and tissues. We discuss their advantages and disadvantages and provide detailed descriptions of the procedures that worked best for plant tissues. We demonstrate that subnanometer structural details are preserved in the process and that meaningful spatial information can be retrieved using rubisco as a model case. Hence, the presented toolbox allows for the first time to do *in situ* structural biology in plant tissues and can likely be adapted to other multicellular systems.

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# Methods

Plunge-frozen and high-pressure frozen samples were prepared with a Vitrobot Mark 4 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and a Leica EM-ICE (Leica Microsystems, Wetzlar, Germany), respectively. Three gallium cryo-focused ion beam (FIB) devices were used: (i) a Scios (Thermo Fisher Scientific, Waltham, MA, U.S.A) equipped with an integrated fluorescence microscope (int-FLM: Meteor (Delmic, Delft, Netherlands), MPLFLN 50x objective (Olympus), NA = 0.8, working distance = 1 mm), (ii) an Aquilos 1 (Thermo Fisher Scientific) with an int-FLM (Meteor (Delmic), LMPLFLN 50x objective (Olympus), NA = 0.5, working distance = 10.6 mm) and an Aquilos 2 (Thermo Fisher Scientific). All FIBs were additionally equipped with a needle micromanipulator (EasyLift, Thermo Fisher Scientific) for lift-out experiments. The procedures described in the following (including stage orientation and milling pattern orientation) are only correct for the standard FIB/SEM geometry employing a shuttle with a pre-tilt of 45° (Extended Data Fig. 2a) and scan rotation for FIB and SEM of 180°.

## *Physcomitrium patens* culture/growth

*Physcomitrium patens* (Hedw.) Gransden ecotype was cultivated on solid BCDAT medium overlaid with cellophane sheets at 25°C and 18h white light/6h dark cycle as described in<sup>35</sup>. Protonemata were harvested after 2 weeks. For plunge-freezing, the tissue was blended in liquid BCDAT medium, allowed to regenerate for 2 – 4 h and then used for cryo-sample preparations. Gametophores were harvested after 4 weeks. For cryo-fixation, individual phyllids or the gametophore tip containing multiple phyllids were cut off, transferred to liquid BCDAT medium and immediately applied to EM-grids or HPF carriers.

## Plunge-freezing and FIB milling of *P. patens* tissues

BCDAT suspended tissues were applied to glow-discharged EM-grids (Quantifoil Cu 200 mesh, holy carbon film R2/1) and transferred to a Vitrobot Mark IV (Thermo Fisher Scientific), temperature set to 25°C and humidity control switched off. Blotting was performed with a Teflon sheet from support film side and a filter paper from the reverse side for 10 s with a blot force 10, before plunging into a liquid ethane-propane mixture<sup>36</sup>.

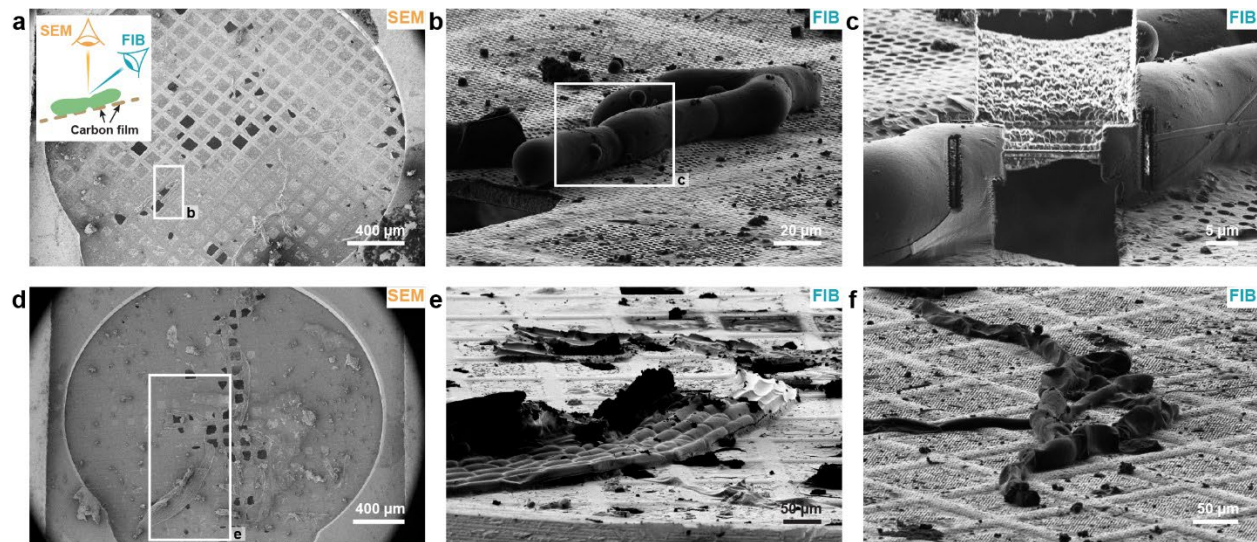
Additionally, different cryoprotectants were tested. Either by culturing *P. patens* on solid BCDAT medium containing cryoprotectants, by incubating *P. patens* after blending in liquid BCDAT medium containing cryoprotectants, by adding 3 µL BCDAT containing cryoprotectants to the EM-grid right before plunging or by combinations of these methods. The different methods with the tested concentrations are listed in Table 1. The use of cryoprotectants either did not prevent the formation of crystalline ice or induced plasmolysis (data not shown) and sometimes even dehydrated the tissue (Extended data Fig. 1f).

**Table 1 | Plunge-freezing of *P. patens* with cryoprotectants.** List of the tested plunge-freezing methods with the employed concentrations of cryoprotectants.

|          | Culture on Solid BCDAT medium with |                      | Incubation in liquid BCDAT medium with |             |           | Before plunging, add 3 µL BCDAT with |                        |
|----------|------------------------------------|----------------------|----------------------------------------|-------------|-----------|--------------------------------------|------------------------|
|          | Cryoprotectant                     | Concentration        | Cryoprot.                              | Conc. (w/V) | Inc. time | Cryoprotectant                       | Concentration          |
| Method 1 |                                    |                      |                                        |             |           | Sucrose                              | 10 %, 15 %, 20 % (w/V) |
| Method 2 | Proline                            | 50 mM, 75 mM, 100 mM |                                        |             |           | Proline                              | 50 mM, 75 mM, 100 mM   |
| Method 3 | Proline                            | 50 mM, 75 mM, 100 mM | Sucrose                                | 10 %, 20 %  | 2 h       |                                      |                        |
| Method 4 |                                    |                      |                                        |             |           | Glycerol                             | 10 % (w/V)             |

Grids were clipped into cartridges with cutouts (Thermo Fisher Scientific) and loaded into the FIB/SEM instrument. A layer of metal organic platinum was applied for 40 s using the gas injection system (GIS) with the stage in the deposition position (here, stage tilt = 25°, stage rotation = 0°, stage z = 12.5 mm, the GIS deposition position may be different for other instruments). Lamellae

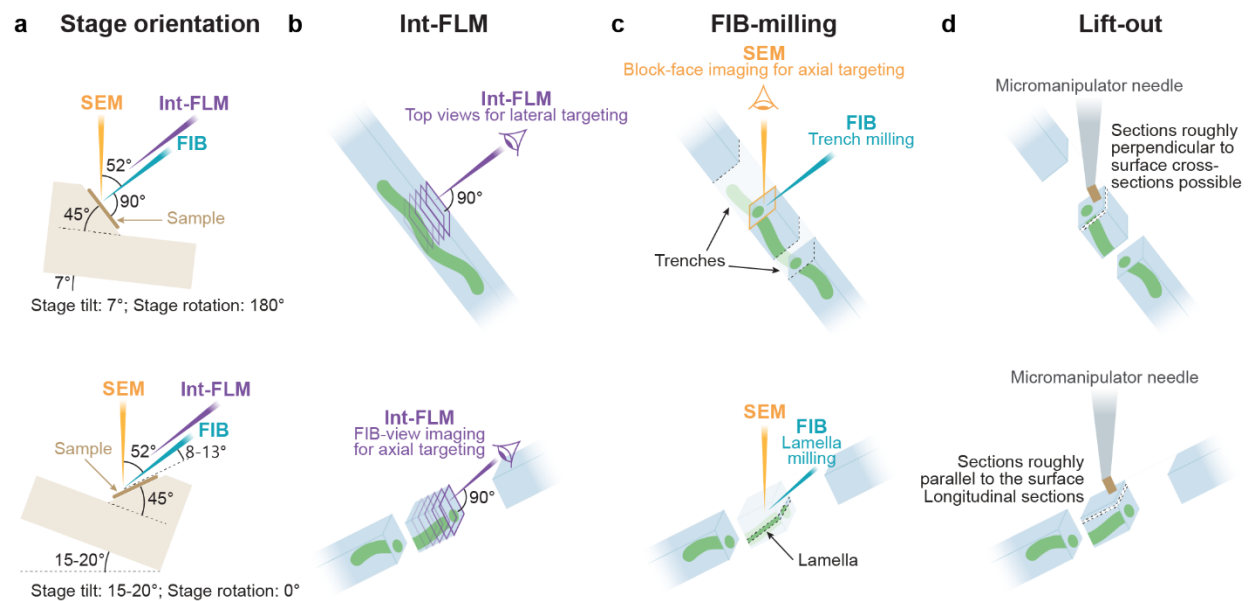
were milled with the stage in lamella milling orientation (stage tilt = 15°, stage rotation = 0°, Extended Data Fig. 2c). This was done in several steps reducing the thickness to 6 µm, 3 µm and 1.5 µm at currents of 1 nA, 0.5 nA and 0.3 nA, respectively. Fine milling was performed at 50 pA, aiming for a final lamella thickness of 200-250 nm. Finally, the lamella was polished at 30 pA at a stage tilt of 14° and 16° only from the bottom and the top, respectively (Extended Data Fig. 1a-d).



**Extended Data Fig. 1 Plunge-frozen *P. Patens* tissues and the use of cryoprotectants.** a-c, Plunge frozen protonemata. a, SEM overview image of a grid. b,c, FIB image before and after lamella milling, respectively. d,e, SEM and FIB images of plunge frozen phyllids, respectively. f, Protonemata grown on medium and plunged in medium containing 100 mM proline as cryoprotectant. The cells are dried out.

## The “waffle” workflow for *Physcomitrium patens* protonemata

Grids were high-pressure frozen and milled using an adapted workflow of the “Waffle” Method<sup>18</sup> similar to the procedure described in<sup>19</sup>. The FIB milling and imaging step described in the following were performed in two main stage orientations. The lamella milling orientation (stage tilt = 15-20°, stage rotation = 0) and trench milling orientation (stage tilt = 7°, stage rotation = 180°). A scheme of the stage orientations is depicted in Extended Data Fig. 2. More details about the workflow can be found in the step-by-step protocol (Pöge *et al.*, will soon be published as a protocol).



**Extended Data Fig. 2 | The different stage orientations used during lamella preparation.** Top and bottom row show the trench milling and lamella milling orientation, respectively, for the geometry in FIB/SEM instruments produced by Thermo Fisher Scientific using a shuttle with 45° pre-tilt. **a**, The shuttle with respect to stage tilt, stage rotation and the relative location of the FIB and SEM beams. **b**, Use of the integrated fluorescence light microscope (int-FLM). In trench milling orientation (top panel), the int-FLM records sample top views for lateral targeting and in lamella milling orientation (bottom panel) it can be used in FIB-view mode for axial targeting. **c**, FIB milling. In trench milling orientation (top panel), the ion beam is normal to the sample surface and used to mill trenches above and below the target area. In Lamella milling orientation (bottom panel), the FIB is used to thin the lamella to a thickness of about 100-300 nm from a shallow angle. **d**, Lift-out. In trench milling orientation (top panel), the lift-out results in sectioning planes roughly normal to the sample surface and cross-sections of protonemata can be obtained. In lamella milling orientation (bottom panel), sectioning planes are roughly parallel to the sample surface resulting in longitudinal sections of protonemata.

## High-pressure freezing of “waffle” grids

As described in<sup>19</sup>, before the experiment, 6mm Type B carriers (300 µm cavity on one side and flat on the other, Leica Microsystems, Wetzlar, Germany) were incubated overnight in a 1:1 mixture of 4 M NaOH and commercial bleach, rinsed 3x with water, 1x with Acetone and dried. Next, the carriers were dipped into a cetyl palmitate solution (0.5-1% (m/v) in diethyl ether), excess of liquid was removed with a filter paper and the carriers were dried on a filter paper with the flat side facing up. Commercially available Copper 50, 75 or 100 mesh grids with continuous support

film (Carbon coated Formvar, Graticules Optics Ltd, Tonbridge, UK) were used to support the sample. As freezing buffer, a 1:1 mixture of growth medium and 40% (m/v) Ficoll 400 (Roth, Karlsruhe, Germany) was prepared. A 2  $\mu$ L droplet was applied to the flat side of a carrier, a grid with the support film facing down placed on it and the excess of buffer removed with a small piece of Whatman No 1 filter paper (Whatman, Maidstone, UK) until the grid was lying flat on the carrier with a thin layer of buffer between them. 3  $\mu$ L buffer were added onto the grid and distributed with the pipet tip until the whole grid surface was covered. Air bubbles were removed with tweezers. Next, a small patch of *P. Patens* protonemata was picked from a plate and placed in the center of the grid. A second carrier was placed on top, flat side down, and the whole sandwich tightly squeezed together for 10 s followed by immediate high-pressure freezing. The resulting “waffle” grids were separated from the carriers, clipped into cartridges with cutouts (Thermo Fisher Scientific) and stored until use in liquid nitrogen (LN<sub>2</sub>).

## Cryo-fluorescence light microscopy

“Waffle” grids have a near featureless surface in SEM and FIB images (Extended Data Fig. 3a). Fluorescence microscopy is therefore vital for targeted lamella preparation. For initial screening, clipped grids were loaded into a confocal laser scanning microscope equipped with cryo-stage (Leica TFS SP8, Leica Microsystems, 50x Air objective: Leica Objective No. 506520, NA 0.9, two hybrid detectors and one photomultiplier tube). We always detected the reflected light (excitation wavelength: 488 nm; emission filter: 480-496 nm), the green autofluorescence of the tissue (excitation wavelength: 488 nm; emission filter: 500-750 nm) and the transmitted light. Dependent on the sample thickness, we adjusted the laser power to have enough signal while not overexposing the detectors. Initially, a grid map was acquired (Extended Data Fig. 3b) with a spiral scan (512x512 pixel per tile image, pin hole size = 600  $\mu$ m, pixel size = 580 nm). Promising grid squares containing cell junctions were subsequently imaged by acquiring z-stacks encompassing the whole sample thickness (2048 x 2048 pixel per image, pin hole size = 125  $\mu$ m,

pixel size = 140 nm, z step size = 1  $\mu$ m, 25-50 steps). Images were recorded using the LAS X software (Leica Microsystems, Wetzlar, Germany). The FLM images displayed in the figures are exclusively single slices of z-stacks and no maximum intensity projections. Squares with several layers of protonemata were ignored because the ~25  $\mu$ m high wells of the used grids can only accommodate a single cell layer risking squeezing damaged during freezing (Extended Data Fig. 3d). Here we used a confocal laser scanning microscope, but a standard widefield system equipped with a cryo-stage could also be used for this screening step.

### Lateral targeting and trench milling

Grids were loaded into a FIB/SEM instrument with integrated fluorescence light microscope (int-FLM). A protective layer of metal-organic platinum was applied for 45 s using the GIS with the stage in trench milling orientation (stage tilt = 7°, stage rotation = 180°, Extended Data Fig. 2). With the stage in trench milling orientation the coincidence point of FIB and SEM was found on a grid square which contained a target area and a marker pattern (cross-section, xyz: 8 x 8 x 0.5  $\mu$ m) was milled at a safe distance from the target area. For lateral targeting, sample top views (Extended Data Fig. 2b top panel) were recorded with the int-FLM using a reflected light channel (excitation wavelength = 485 nm, no emission filter) to focus on the surface and to find the marker and a green-fluorescence channel (excitation wavelength = 485 nm, emission wavelength = 525 nm). The junctions between *P. patens* cells could be detected based on the green cellular autofluorescence without additional labelling. Z-stacks were acquired (pixel size = 168 nm, 20-25 z-slices, z-step = 1  $\mu$ m). The distance as well as the location of the target area with respect to the marker was determined (Extended Data Fig. 3f) using the Odemis software (Delmic). Dependent on the sample thickness, the laser power was adjusted to have enough signal to detect the target area but kept at the bare minimum to not melt the sample.



centered. First, a marker pattern was milled (yellow arrowhead) and identified in int-FLM images. From there the distance to the target area was measured (white arrowhead) in (f). This information was used to place the patterns for milling step I in (e). The results are shown in (g). After trench milling the target area is centered within the block between trenches (h). In (f)-(g) white rectangles indicate the field of view in the second image.

Based on the measured distance of the target area from the marker, the position of trench milling patterns were defined. The drift suppression function of the instrument was activated during all trench milling steps. First, two cross-sections with an xy-size of 40x30  $\mu\text{m}$  were milled above and below the target area with a distance of 40  $\mu\text{m}$  at an ion current of 7 nA (step I in Extended Data Fig. 3e). Next, the trenches were elongated at 15 nA to a height of 50  $\mu\text{m}$  and 80-120  $\mu\text{m}$  (step II in Extended Data Fig. 3e), on bottom and top, respectively. The target area containing block was further thinned in two steps to an xy-size of 35x30  $\mu\text{m}$  and 30x20  $\mu\text{m}$  at 5 nA and 3 nA, respectively (step III and IV in Extended Data Fig. 3e). Top views were acquired with FIB and int-FLM after each milling step to adjust the positioning of the patterns, assuring that the targeted area was centered after trench milling (Extended Data Fig. 3g-h). Then the block-face on top was polished at 1 nA.

## Axial targeting

The images of the int-FLM allow to reliably localize the target area laterally, i.e. in parallel to the sample surface. Due to the limited numerical aperture of the objective, the wide-field epifluorescence optical setup of the int-FLM and depth dependent aberrations, the axial resolution is limited. The axial localization of the target area within the thickness of the sample is crucial to place the patterns for lamella fine milling. Two methods were used for the axial targeting: (i) SEM block-face imaging and (ii) FIB-view imaging with the int-FLM.

After trench milling, with the stage still in trench milling orientation, the SEM images the side surface of the block (Extended Data Fig. 2c, top panel). Cellular structures, like membranes, exposed at the FIB milled front of the block, can be visualized with by SEM imaging, even for non-

metal stained, vitrified samples<sup>37</sup>. Before the image was recorded, the auto-stigmator function was run on a nearby spherical contamination. The whole block-face was then centered in the SEM field-of-view, the SEM focused on the edge between grid surface and block-face and the auto brightness-contrast function run on the block-face itself. SEM images were acquired with a dwell time of 50 ns, 100 line integrations at U = 3 kV, I = 13 pA with an image size of 3072 x 2048 pixel (Figure 2d). If inherent material contrast is sufficiently strong, the approximate distance of the target area from the sample surface can then be measured in the SEM image using the FIB/SEM user interface.

For FIB-view imaging, the int-FLM was used to image the side of the block after trench milling in lamella milling orientation (Extended Data Fig. 2b, bottom panel) at the angle at which the lamella will be milled. For optimal imaging, the lamella front has to be normal to the optical path of the int-FLM (Capitanio *et al.*, will soon be described in a manuscript). This allows to avoid major depth-dependent refractive-index-mismatch-induced aberrations<sup>38</sup>. Normal incidence is achieved by changing the stage tilt angle during trench milling steps III-IV (Extended Data Fig. 3e). The required stage tilt depends on the stage tilt required for FIB-view imaging which, in turn, is dependent on the stage tilt for lamella fine milling. For waffle milling, FIB-view images were recorded at a stage tilt of 20° in lamella milling orientation, requiring trench milling steps III-IV at -6° stage tilt. The required stage tilt for trench milling steps III-IV ( $\alpha_{\text{Trench milling}}$ ) as a function of the stage tilt for FIB-view imaging ( $\alpha_{\text{FIB-view}}$ ) can be calculated according to this equation:

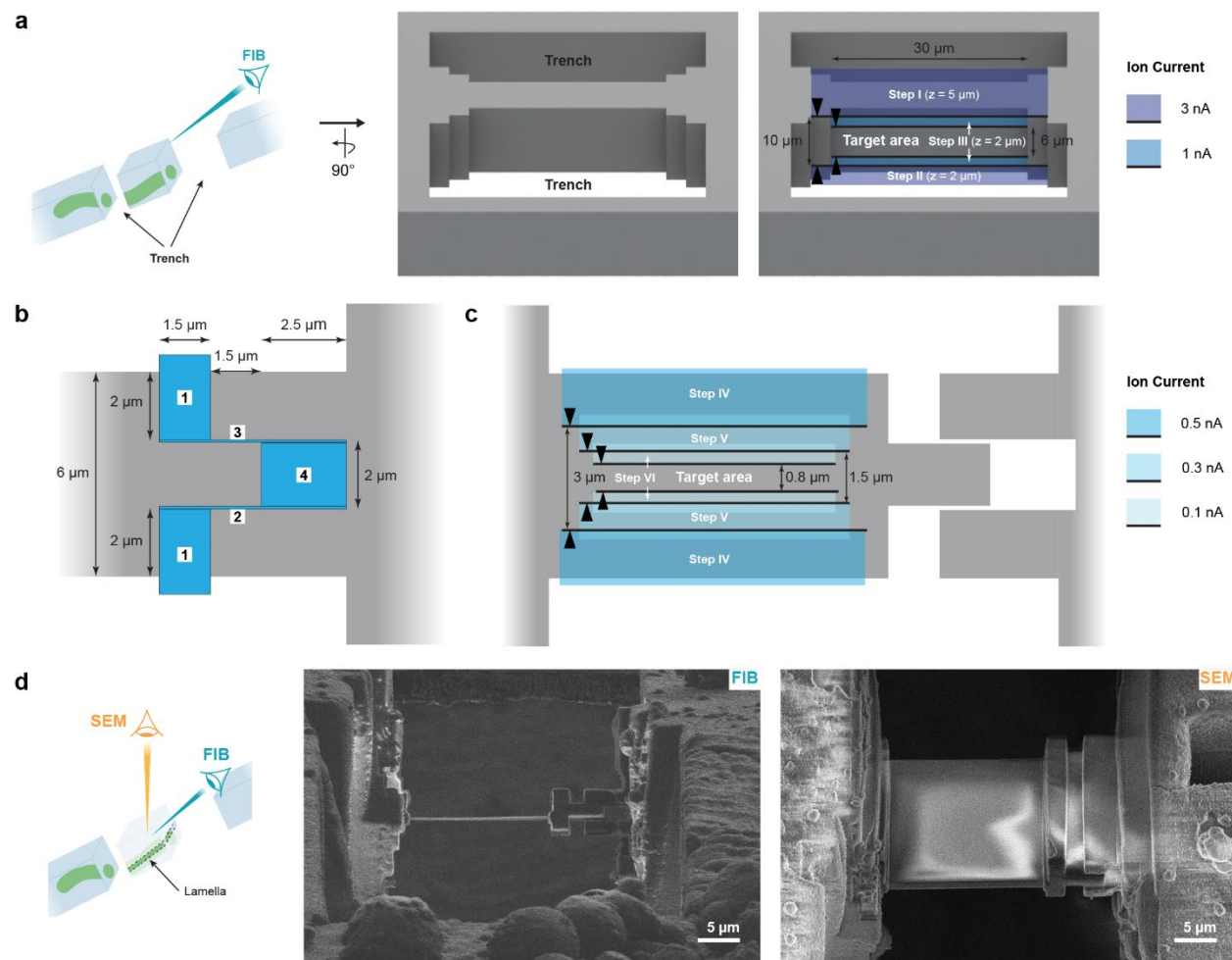
$$\alpha_{\text{Trench milling}} = -\alpha_{\text{FIB-view}} + 14^\circ$$

This equation is only valid for the standard geometry used in FIB/SEM instruments employing a shuttle with a pre-tilt of 45°. Care must be taken that the difference between trench milling and FIB-view imaging is not only the stage tilt but also the stage rotation. Trench milling is performed with a stage rotation of 180° and FIB-view imaging with a stage rotation of 0° (Extended Data Fig. 2).

First, the lamella front and the target area were identified in the reflected light channel (excitation wavelength = 485 nm, no emission filter) and the green-fluorescence channel (excitation wavelength = 485 nm, emission wavelength = 525 nm), respectively and z-stack recorded (pixel size = 168 nm, 20 z-slices, z-step = 1  $\mu$ m) using the Odemis software (Delmic). To target the junction between cells, it was sufficient to roughly measure its distance from the sample surface in the Odemis software. For smaller targets, the lamella front can be protected by a layer of metal organic platinum using the GIS and reference marker milled as described (Capitanio *et al.*, will soon be described in a manuscript)

# Fine milling of “waffle” lamellae

The surfaces of the block after trench milling were coated with a thick layer of metal organic platinum using the GIS in trench milling orientation and in lamella milling orientation for 15 s and 3 times 20 s, respectively. Fine milling was performed at a stage tilt of 20° in lamella milling orientation (Extended Data Fig. 2c). First, all material below the target area was removed at an ion current of 3 nA. Next, regular cross-sections were used to thin the lamella to an xy-size of 30x10  $\mu$ m with a beam current of 3 nA and then down to 30x6  $\mu$ m with 1 nA (Extended Data Fig. 4a). At this point, a notch similar to<sup>18</sup>, but larger, was introduced at current of 0.5 nA (Extended Data Fig. 4b). Subsequently, the width of the milling patterns was reduced in x to 18-20  $\mu$ m and the lamella thinned at 0.5 nA to 3  $\mu$ m, 0.3 nA to 1.5  $\mu$ m and 0.1 nA to 0.8  $\mu$ m using regular cross-section patterns (Extended Data Fig. 4c). Fine milling was performed with rectangle patterns milled in parallel, aiming for a lamella thickness of 250 nm at 50 pA. Finally, the stage was tilted to 19° and 20.5-21°, removing material only on bottom and top, respectively, at 30 pA.



**Extended Data Fig. 4 | Fine milling of “waffle” lamellae.** **a-c**, Scheme of the milling patterns used for “waffle” lamella preparation. The block after trench milling and rotating the stage into the lamella milling orientation is shown in **(a)**. First the lamella is thinned to a thickness of 6 μm. Then a broad notch is milled **(b)** and the lamella further thinned down **(c)**. All thinning steps in **(a)** and **(c)** were milled with regular cross-section with milling direction towards the target area indicated by black arrowheads. Fine milling was then performed with rectangle patterns milled in parallel. **d**, FIB and SEM image of a “waffle” lamella.

## Serial Lift-Out

The EasyLift system (Thermo Fisher Scientific) installed on a Scios and an Aquilos 2 was used for the lift-out experiments. Prior to the lift-out, a copper adapter was attached to the needle of the EasyLift system as described in<sup>19</sup>.

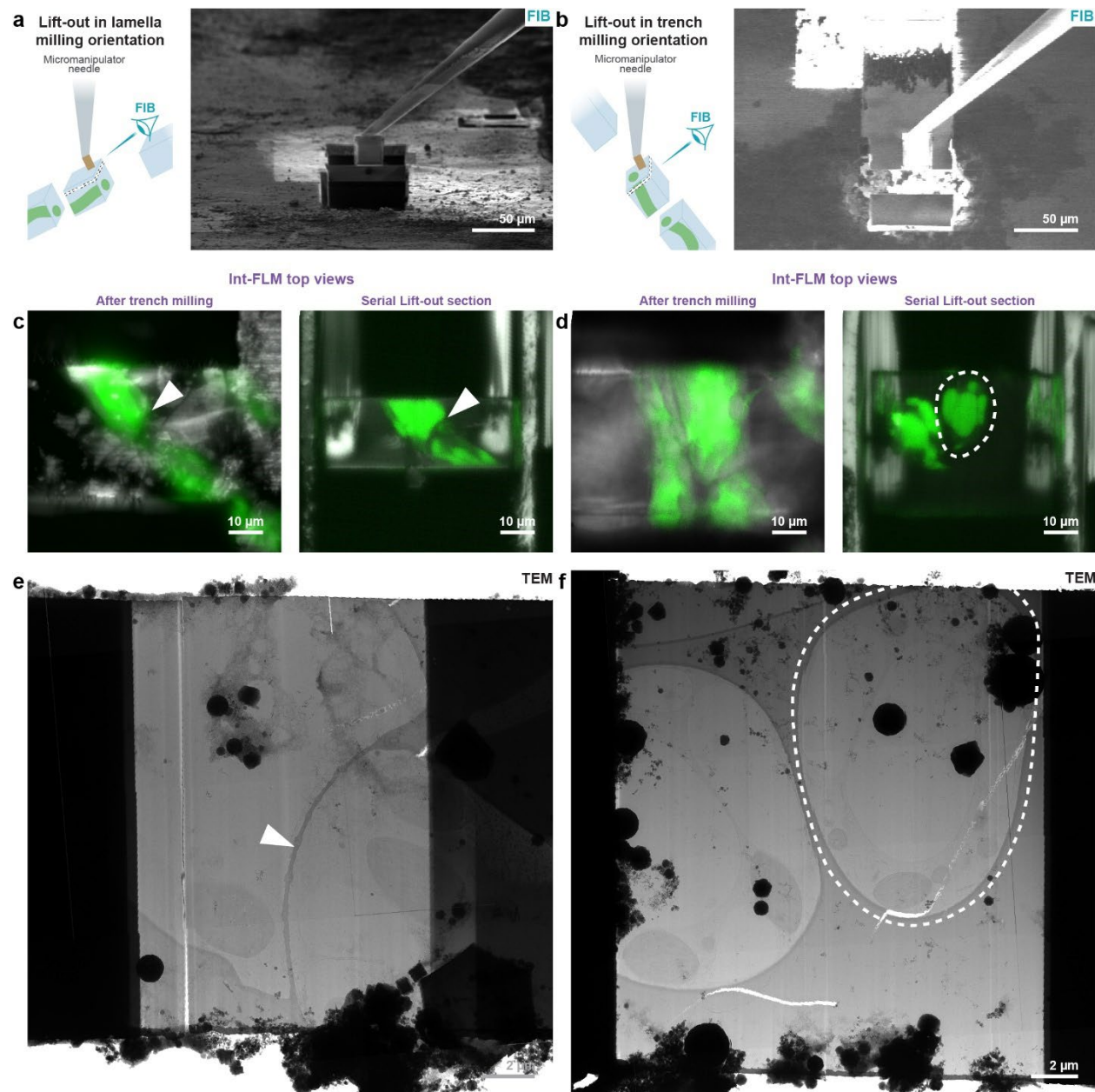
## Lift-out

The first step of the lift-out experiment was targeted trench milling, as described for “waffle” milling in the previous sections. The width of the block between the trenches depends on the size of the target area. The trenches, however, should be roughly 10  $\mu\text{m}$  wider than the desired width of the final lift-out block as it must be detached from the bulk sample on both sides.

We use redeposition milling to attach a copper adapter to the needle of the micromanipulator, the lift-out block to the copper adapter and the sections to the receiver grid. The pattern used for redeposition milling is an array of regular cross-section with lateral dimensions of 0.5 x 2.5  $\mu\text{m}$  at a distance of 0.25  $\mu\text{m}$  with the multi-pass option set to one. The cross-section patterns are placed along the whole interface of the surfaces that are being attached, on the copper face and the 2.5  $\mu\text{m}$  long dimension is always perpendicular to the attached surface. The milling direction is always away from the interface and the z-dimension of all cross-sections set to 4  $\mu\text{m}$ . On FIB/SEM instruments with imperfect ion beam optics alignment, it can be beneficial to make increase the size of the cross-sections and the distance between them by 40 – 60 % in all directions. A more detailed description of the milling patterns and the whole workflow can be found in the step-by-step protocol (Pöge *et al.*, will soon be published as a protocol).

We used two distinct lift-out orientations: (i) from the trench milling orientation, the lift-out direction is roughly in the plane of the sample surface or (ii) from the lamella milling orientation where it is roughly perpendicular to the sample surface (Extended Data Fig. 2d). For (i) the copper adapter was positioned in the center of the block-face directly after trench milling (Extended Data Fig. 5b). Afterwards, the lift-out was performed as described in<sup>19</sup>. For (ii), after trench milling the stage was moved to the lamella milling orientation and a thick layer of metal organic platinum applied by opening the GIS for 3x20 s. Next, all material above and below the target area was removed at 3 nA and the top of the block polished at 1 nA. Then, the copper adapter was positioned in the center on top of the lift-out block and redeposition milling was used to attach it to the lift-out block

(Extended Data Fig. 5a). Only then was the lift-out block detached from the bulk sample on both sides and subsequently extracted.

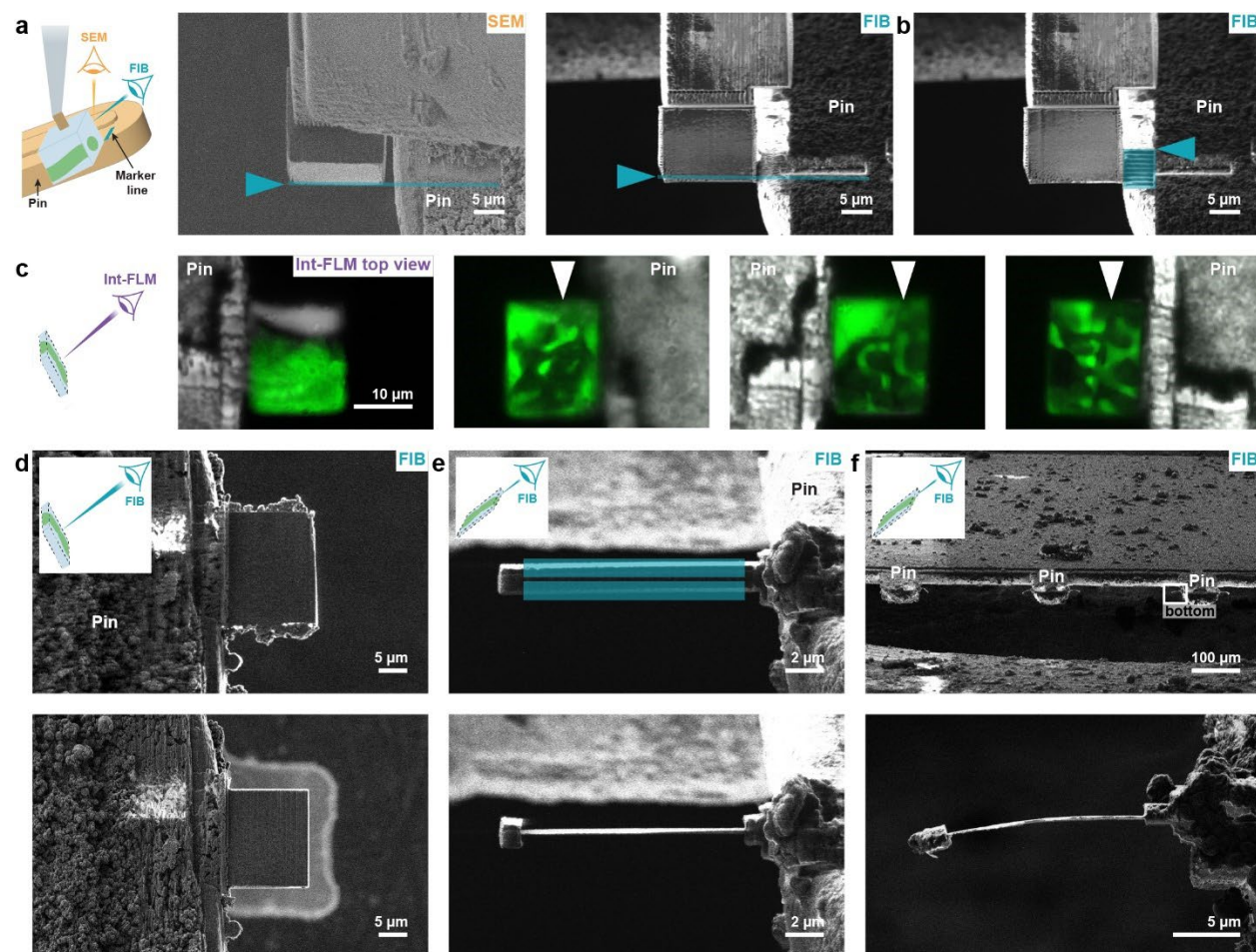


**Extended Data Fig. 5 | Lift-out in different stage orientations.** The left panel shows data for the lift-out with the stage in lamella milling orientation, the right panel for the lift-out in trench milling orientation. **a,b**, FIB images with the needle attached to the lift-out block. **c,d**, Int-FLM top views. The left image and the right image were recorded after trench milling and after sectioning, respectively (reflected light in gray and cellular autofluorescence in green). **e,f**, TEM overviews of the final lamellae obtained from the sections in (c) and (d). The outlines of protonemata cross-sections are marked by dashed lines and cell junctions by white arrowheads.

Lift-out from different stage orientations can help imaging cells with preferred orientation on the grid from different directions. Lift-out in trench milling orientation results in cross-sections of protonemata if the axis of the filament runs parallel to the lift-out direction (Extended Data Fig. 5c,e).. In contrast lift-out in lamella milling orientation results in longitudinal sections of protonemata (Extended Data Fig. 5d,f).

## Lift-in using single-sided attachment

For the single-sided lift-in attachment, copper 4-pin half-moon grids (Pelco FIB-Lift-Out-Grids, Plano GmbH, Wetzlar, Germany) were used as receiver grids. After loading, the grid was rotated into trench milling orientation and marker lines milled on either side at the tip of each pin. After rotating back to lamella milling orientation, the needle, with the lift-out block attached, was reinserted and the lower front edge of the lift-out block was aligned to the marker line (Extended Data Fig. 6a). Redeposition milling at 1 nA was used to attach the bottom part of the lift-out block to the pin (Extended Data Fig. 6b). The needle was then moved 200 nm up in z to apply some strain. A horizontal line pattern was milled to section the lowest 3-4  $\mu\text{m}$  of the lift-out block and a vertical line pattern above the attachment patterns at the interface between lift-out block and pin used to fully detach the remaining lift-out block. This process of attachment and sectioning was repeated until the lift-out block was sliced up. More details about the exact milling patterns can be found in the step-by-step protocol (Pöge *et al.*, will soon be published as a protocol).



**Extended Data Fig. 6 | Serial Lift-Out with single-sided attachment.** **a**, Alignment of the lift-out block to the marker line (blue line and arrowhead) in FIB and SEM. **b**, Attachment of the lower part of the lift-out block on one side. The patterns for redeposition milling are indicated in blue. **c**, Int-FLM top views of single-sided attached Serial Lift-Out sections acquired in trench milling orientation (reflected light in gray, cellular autofluorescence in green). The junction between cells is only contained the three right-most sections, marked by white arrowheads. **d**, Section trimming in trench milling orientation. A section before and after trimming is shown in the top and bottom panel, respectively. **e**, Single-sided attached lamella before and after fine milling in the top and bottom panel, respectively. The milling patterns are indicated in blue. A 1.5x1.5  $\mu\text{m}$  block is left opposite of the attachment site. **f**, Single-sided attached lamellae often bend during or after fine milling.

## Section trimming

For each section, top views images were recorded with the int-FLM in trench milling orientation to identify sections containing the target area (Extended Data Fig. 6c). Next, the GIS was used for 20 s in trench milling orientation. The section was subsequently trimmed down at 3 nA to

primarily contain the target area (Extended Data Fig. 6d), and the front edge on top was polished at 1 nA. To protect the new lamella front, the GIS was used for 15 s and 3x20 s in trench milling and lamella milling orientation, respectively. This trimming step minimizes the size of the lamella and generates a smooth, coated lamella front, which makes the subsequent fine milling faster and easier.

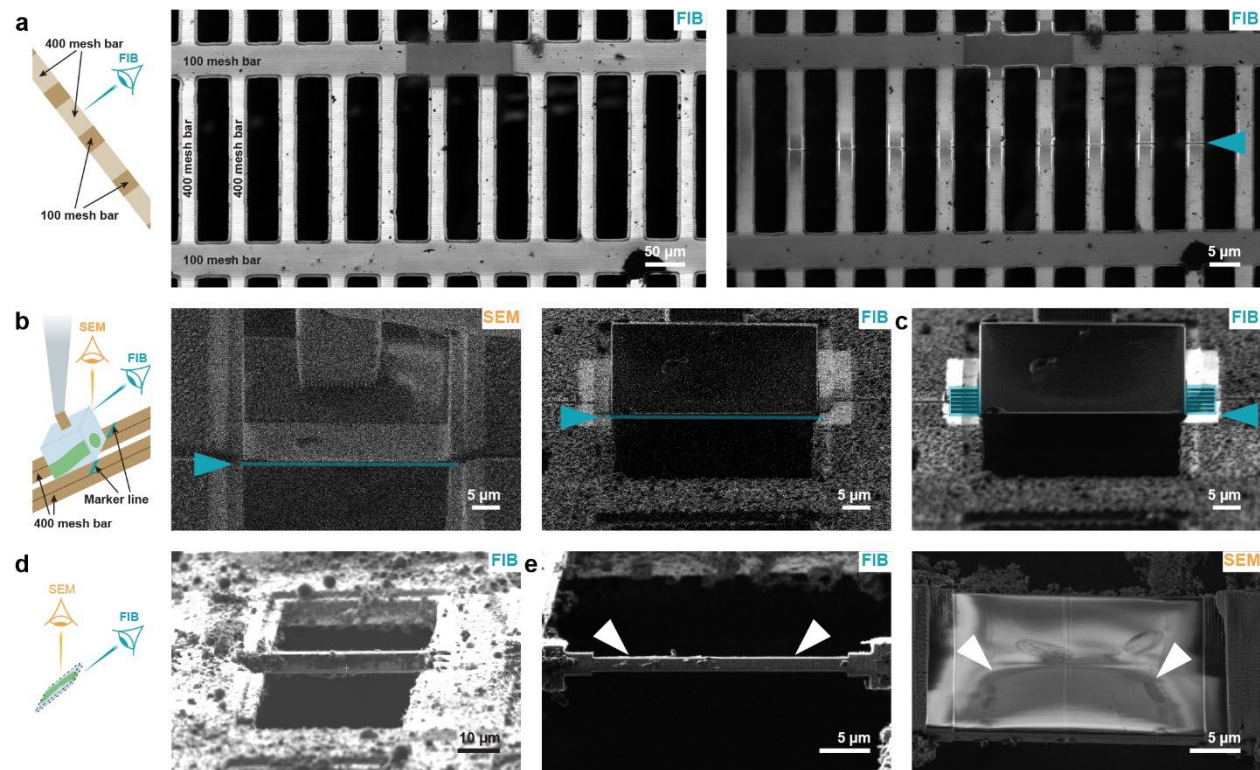
## Fine milling of lamellae with single-sided attachment

The sections were thinned in lamella milling orientation (stage tilt = 15°) at 1 nA to 2.5 µm and at 0.3 nA to 1.5 µm using regular cross-section patterns. Opposite of the attachment site, a 1.5x1.5 µm block was left which prevents folding<sup>39</sup> of the lamella (Extended Data Fig. 6e). Fine milling was done at 50 pA using rectangle patterns milled in parallelset at a distance of 250 nm from one another, followed by tilting to 14° and 15.5°-16° while milling only on bottom and top, respectively, at 30 pA. Lamellae with single-sided attachment had the tendency to bend (Extended Data Fig. 6f) making fine milling difficult. Additionally, they often broke during transfers into the TEM and displayed sample movement during data acquisition. By reducing the width of the lamella from the attachment site and increasing its depth along the attachment site, this could be reduced but not completely prevented, especially for lamellae thinner than 200 nm. Thus, we strongly recommend using the double-sided attachment procedure as described below.

## Lift-in using double-sided attachment from the sides

For the double-sided attachment, as described in<sup>19</sup>, the lift-out block must fit precisely between two neighboring 400 mesh bars of a 400x100 mesh grid (~40 µm). As such, the trench milling patterns were adjusted so that the final lift-out block had a width of 42-45 µm. When mounting the 400x100 mesh grid into the shuttle, it is crucial that the 100 mesh bars are aligned horizontally. Marker lines were milled in trench milling orientation in the middle of 400 mesh bars (Extended Data Fig. 7a). Lift-in was performed in lamella milling orientation (stage tilt = 15°). After precise

vertical alignment of the 400 mesh bars by rotating the stage, the width of the lift-out block was adjusted to fit precisely between them by FIB milling using cross-sections at 1 nA. The lower front edge of the lift-out block was then aligned to the marker lines of the grid bars in the FIB and SEM channels (Extended Data Fig. 7b).



**Extended Data Fig. 7 | Serial Lift-Out with double-sided attachment from the sides.** **a**, Milling of marker lines for the alignment of the lift-out block. Grid bars before and after marker line milling in the left and right panel, respectively. Marker lines are indicated by a blue arrowhead. **b**, Alignment of the lift-out block to the marker lines. SEM and FIB images are shown in the left and right panel, respectively. The bottom front edge of the lift-out block is indicated by a blue line and the marker line by a blue arrowhead. **c**, Attachment of the lift-out block on both sides. Patterns for redeposition milling are indicated in blue. **d**, FIB image of a lift-out section. **e**, FIB and SEM image of the section in the left and right panel, respectively, after the top layer was removed. The SEM image reveals the location of the cell junction as target area (white arrowheads) which was, subsequently, used to minimize the width of the milling patterns for lamella fine milling.

Redeposition milling at 1 nA was used to attach the lower part of the lift-out block to the grid bars on both sides (Extended Data Fig. 7c). Then, the needle was moved up in z by 200 nm. A horizontal line pattern was milled to detach the lower 4  $\mu\text{m}$  of the lift-out block and two vertical

line patterns were milled on both sides above the attachment patterns at the interface between lift-out block and grid bars to fully detach the remaining lift-out-block. This process of attachment and sectioning was repeated until the whole lift-out block was sliced up. More details about the exact milling patterns can be found in the step-by-step protocol (Pöge *et al.*, will soon be published as a protocol).

Similar to single sided attachment, after sectioning, top views were recorded for each lamella precursor with the int-FLM in trench milling orientation. Regions that did not contain the target area were removed at 3 nA and the front edge on top was polished at 1 nA (Figure 4). To protect the new lamella front, the GIS was used for 15 s and 3x20 s in trench milling in lamella milling orientation, respectively.

Fine milling was performed in lamella milling orientation (stage tilt = 15°) in several steps. First, the top of the section was removed at 1 nA. Subsequently, the lateral location of the target area was determined based on either the int-FLM top views recorded prior to section trimming or an SEM block-face image (Extended Data Fig. 7e). Using this information, the milling patterns were centered around the target area to reduce the width of the lamella to a minimum. Afterwards, sections were thinned at 1 nA to 2.5 µm, 0.3 nA to 1.5 µm and 0.1 nA to 0.8 µm using regular cross-section patterns. Fine milling was done at 50 pA using rectangle patterns with a distance of 250 nm milled in parallel, followed by tilting to 14° and 15.5°-16° while milling only on bottom and top, respectively, at 30 pA.

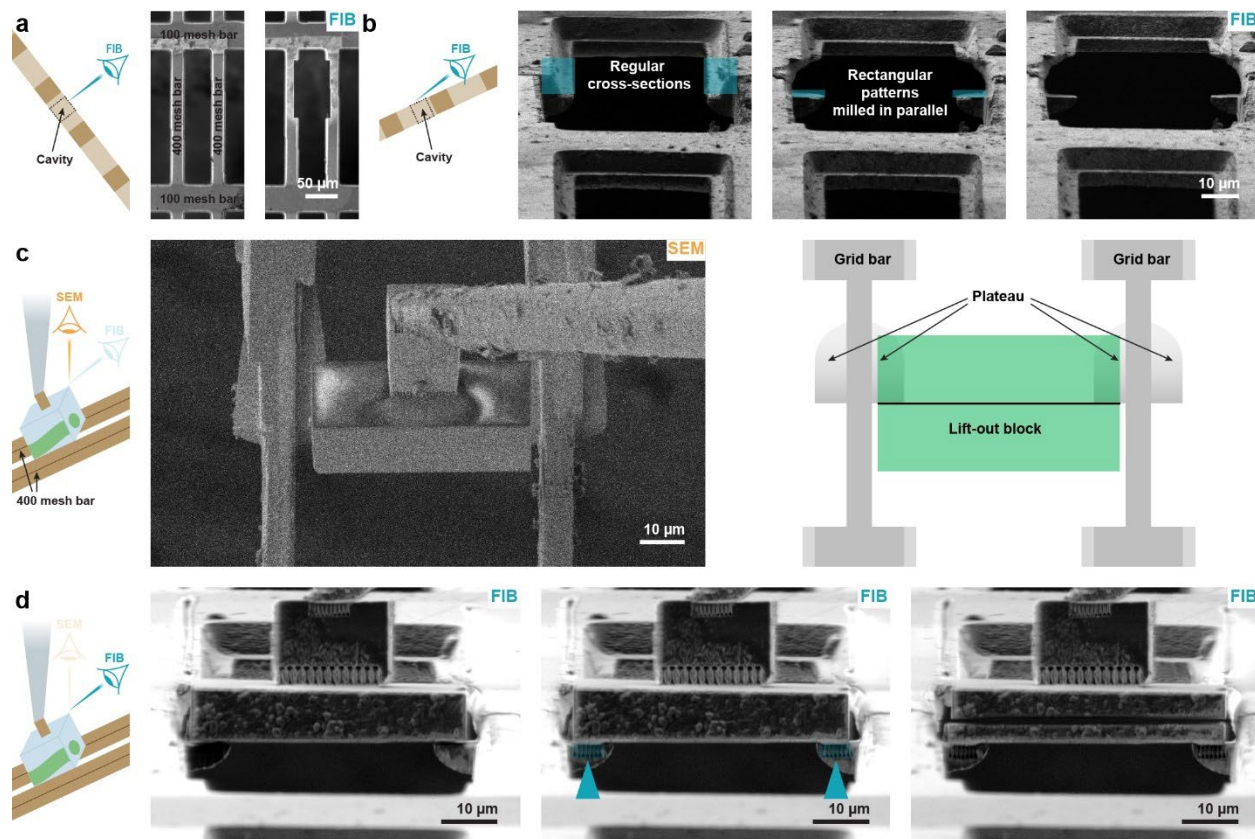
### Lift-in using double-sided attachment from below

The idea of the attachment from below is to mill two plateaus into neighboring grid bars, both at the same height and parallel to the bottom of the lift-out block. Thus, the lift-out block can be placed on the plateaus instead of being placed between grid bars. Before the lift-out experiment, a 400x100 mesh grid was loaded into the FIB at room temperature and modified. In trench milling

orientation, with an ion current of 64 nA, 8x90  $\mu\text{m}$  wide (xy-size) rectangular cavities were milled into the neighboring 400 mesh bars with the lower end of the patterns in the middle of rectangle (Extended Data Fig. 8a). The modified grids can be stored until further use.

For the lift-out experiment, a modified 400x100 mesh grid was loaded with the 100 mesh bars aligned horizontally in lamella milling orientation (stage tilt 15°). Before the first Serial Lift-Out sections could be attached, the actual plateaus were milled in the back of the cavities (Extended Data Fig. 8b). First, regular cross-section patterns were milled, preparing the rough plateaus, followed by two rectangle patterns milled in parallel on either side to assure that the plateaus have the same height. The width of the resulting attachment site is between 50-55  $\mu\text{m}$ . The lift-out block must be 1-2  $\mu\text{m}$  slimmer to fit in the gap but must still overlap several micrometers with the plateaus on both sides. This was achieved by adjusting the width of lift-out block during trench milling.

After the lift-out, the bottom of the lift-out block was polished resulting in a surface parallel to the plateaus. Next, the center of the lift-out block was aligned to the front edge of the plateaus (Extended Data Fig. 8c). Redeposition milling was used to attach the lower part of the lift-out block on both sides to the plateaus (Extended Data Fig. 8d). After the attachment, the needle was moved up in z by 200 nm and a line pattern was used to detach the lowest 3  $\mu\text{m}$  of the lift-out block. This process of attachment and sectioning was repeated until the whole lift-out block was sliced up. Afterwards, top views were recorded with the int-FLM for each section followed by trimming and fine milling as described in the previous section.

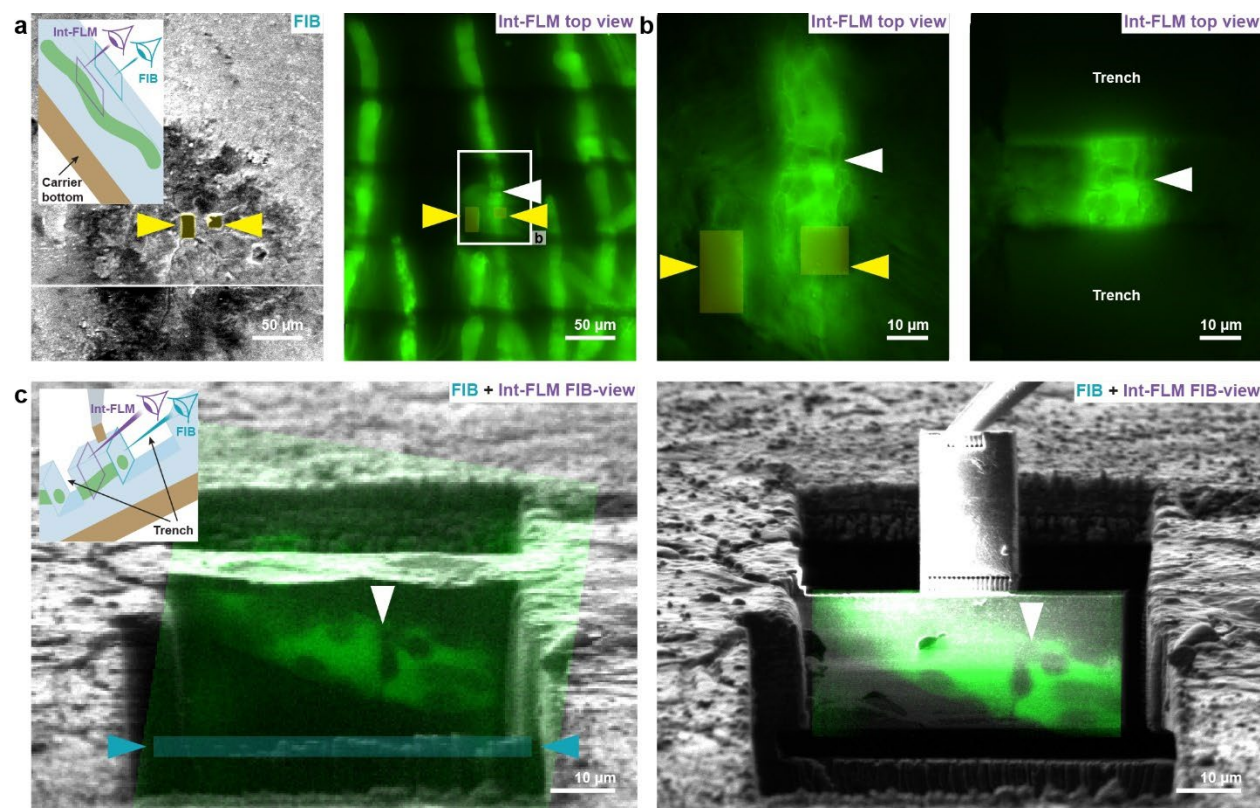


**Extended Data Fig. 8 | Serial Lift-Out with double-sided attachment from below.** **a**, Preparation of 8x90  $\mu\text{m}$  cavities in trench milling orientation. **b**, Preparation of the plateaus in lamella milling orientation. Left panel: FIB image of cavities before milling. Middle panel: after milling regular cross-section patterns (xyz: 9 x 8 x 20  $\mu\text{m}$ ) generating the rough shape of the plateaus. Due to stage drift, they can have different heights. Right panel: After rectangle patterns were milled in parallel (xyz: 9 x 1 x 20  $\mu\text{m}$ ) to even out height differences. This allows the attachment of the bottom of the lift-out block to both plateaus from below. Milling patterns are indicated by blue rectangles. **c**, Alignment of the lift-out block with respect to the plateaus in the SEM. The center of the block should be roughly on top of the front edge of the plateaus (sketch in right panel). Precision of the placement, however, is not imperative. **d**, the lift-out block placed on the plateaus, after attachment from below and after sectioning in the left, middle and right panel, respectively. Patterns for redeposition milling are depicted in blue.

## In-carrier high-pressure freezing for *P. patens*

Commercially available 3mm Type A and Type B carriers (Leica Microsystems, Wetzlar, Germany) were used for in-carrier HPF. Type A has a 100  $\mu\text{m}$  deep well on one side and a 200  $\mu\text{m}$  well on the other. Type B has only a single 300  $\mu\text{m}$  deep well and is flat on the other side. Carriers were incubated overnight in a 1:1 mixture of 4 M NaOH and commercial bleach, rinsed 3x with

water, 1x with Acetone and dried. Subsequently, the type only B carriers were coated by dipping them into cetyl palmitate solution (0.5 (m/v) in diethyl ether), removing excess of liquid with filter paper and drying carriers with the flat side facing up on filter paper. As freezing buffer, a 1:1 mixture of growth medium and 40% Ficoll 400 was used. The 100  $\mu\text{m}$  well of the type A carrier was filled with 2  $\mu\text{L}$  freezing buffer and the droplet spread over the entire well with the pipet tip. A small patch of *P. patens* tissue was placed in the droplet, air bubbles were removed with tweezers and the excess of buffer removed with filter paper until the meniscus of the droplet almost vanished. The type B carrier was placed on top with the flat side down, the sandwich squeezed together and immediately high-pressure frozen. Afterwards, the sample containing type A carriers were recovered and stored until further use.



**Extended Data Fig. 9 | Preparation of in-carrier frozen HPF samples for lift-out.** **a**, Screening of in-carrier frozen HPF sample using the int-FLM. FIB and a montage of int-FLM top view images in left and right panel, respectively. The field-of-view in **(b)** is marked by a white rectangle. **b**, Int-FLM images before and after trench milling in the left and right panel, respectively. In **(a)** and **(b)**, marker patterns are labelled with yellow arrowheads and yellow rectangles. **c**, Milling of the undercut, aided by the int-FLM for in-carrier frozen

samples. Overlay of FIB images (gray) and int-FLM FIB-view image of cellular autofluorescence (green) before and after milling the undercut in the left and right panel, respectively. Both images were recorded in lamella milling orientation. The undercut is marked by a blue rectangle. In (a)-(c) cell junctions are labelled by white arrowheads.

In-carrier HPF samples were not screened in our FLM as they don not fit into the commercial shuttle. Hence, they were screened using the int-FLM. After loading the samples into the FIB, the sample surface was protected by a layer of metal organic platinum for 45 s by using the GIS with the stage in trench milling orientation. Afterwards, marker patterns were milled (xy-dimensions: 6 x 16  $\mu\text{m}$  and 8 x 8  $\mu\text{m}$  at a distance of  $\sim 10 \mu\text{m}$  with z-dimension = 0.5  $\mu\text{m}$ ) and top view images were recorded with the int-FLM, covering area of 1000  $\mu\text{m}$  x 1000  $\mu\text{m}$  around the marker pattern (Extended Data Fig. 9a). Targeted trench milling was performed as described for “waffle” grids (Extended Data Fig. 9b) with the exception that target areas were also selected based on their proximity to the sample surface, which could be roughly estimated based on where the fluorescence was localized within the acquired int-FLM z-stacks.

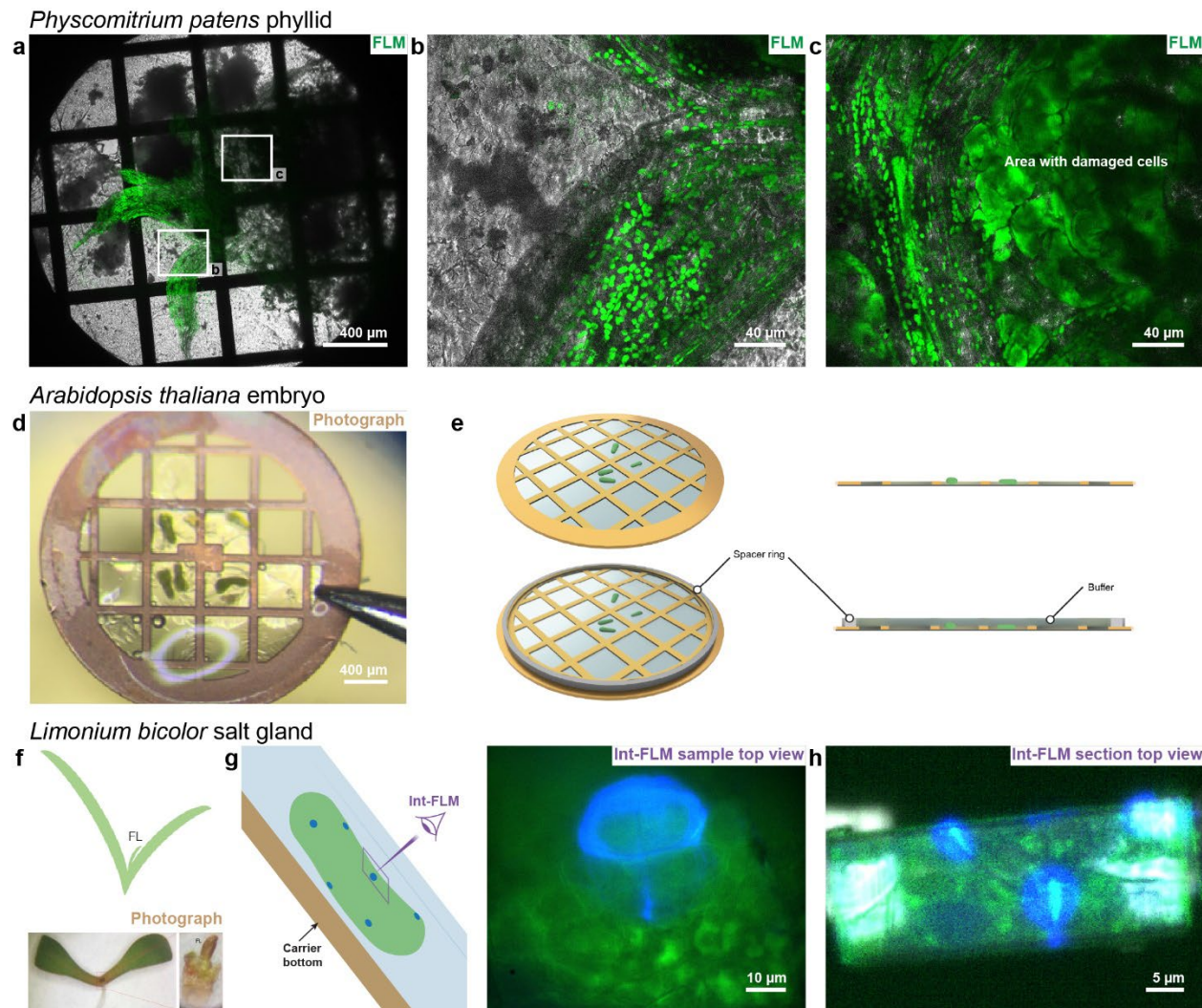
To fully detach the lift-out block from an in-carrier HPF sample, an additional undercut is necessary. First, the axial location of the target area was determined either by FIB-view imaging with the int-FLM (Extended Data Fig. 9c) or SEM block-face imaging (Fig. 2d). With the stage in lamella milling orientation, the front of the lift-out block protected by metal organic platinum and the undercut milled below the target area at 3 nA. Afterwards, the lift-out was performed as described in the previous section either from the trench milling or the lamella milling orientation.

## Lamella sputter coating after fine milling

Lamellae were sputter coated after fine milling. The sputtering systems on the used Aquilos 1 and Aquilos 2 introduce 5-15 nm sized spheroidal platinum particles onto the lamella surfaces. The sputtering conditions were set to  $U = 1 \text{ kV}$ ,  $p = 0.1\text{-}0.2 \text{ mbar}$ ,  $I = 10\text{-}15 \text{ mA}$ ,  $t = 2\text{-}4 \text{ s}$ . These particles aid frame and tilt-series alignment during TEM data processing. The fiducial based tilt-series alignment resulted in higher quality tomograms than patch tracking approaches.

## “Waffle” workflow for *P. patens* phyllids

The “waffle” grids of *P. patens* phyllids were prepared similar to protonemata. A cleaned and cetyl palmitate coated 6 mm type B carrier was placed with the flat side facing up on filter paper and a 3  $\mu$ L droplet of freezing buffer placed on it. Next, a 50 mesh EM-grid with continuous support film (Carbon coated Formvar, Graticules Optics Ltd, Tonbridge, UK) was placed on the droplet with the support side facing down and excess of buffer removed until the grid was resting flat on the carrier. A 3  $\mu$ L droplet of freezing buffer was added on top of the grid and distributed to fill all wells formed by support film and grid bars and air bubbles removed with sharp tweezers. A small piece of phyllid was cut from the gametophore with fine surgical tools and placed in the center of the grid. A second coated 6 mm type B carrier was placed with the flat side facing down on top, the sandwich squeezed together and excess of buffer removed with a filter paper. Next the sandwich was mounted for HPF and squeezed tightly for 10 s right before freezing. Areas of tissue that were damaged in the process could be identified in the FLM (Extended Data Fig. 10a-c). In areas with largely intact cells, individual chloroplasts can be resolved (Extended Data Fig. 10b), whereas for damaged cells, the green autofluorescence signal is diffusely distributed throughout the whole cell (Extended Data Fig. 10c). Lamellae were otherwise prepared by “waffle” milling or lift-out as described in the previous sections.



**Extended Data Fig. 10 | HPF workflows applied to other tissues and species. a-c, *Physcomitrium patens* phyllids. a, FLM overview of a phyllid "waffle" grid. b, Grid square containing seemingly intact tissue. c, Grid square containing damaged tissue. d, *Arabidopsis thaliana* embryos. d, Image of embryos placed in the squares of a 50 mesh grid. e, Schematic of a "waffle" grid with and without a spacer ring. Embryos fit into grid squares but are thicker than the grid bars and would therefore be damaged during freezing (top panel). The addition of a spacer ring, as described in<sup>18</sup>, prevents squeezing damage and can allow high-pressure freezing of thicker samples. f-h, Salt gland of *Limonium bicolor*. f, Dissection of the first true leaf (FL). The top panel shows a scheme of the FL location in 2 weeks old plants. The bottom panel are photographs of a whole two weeks old plant before and the dissected FL in the left and right panel, respectively. g, int-FLM top view image of the salt gland of in-carrier frozen FL. h, int-FLM image top view image of a Serial Lift-Out section containing a fraction of the salt gland. In (g) and (h) autofluorescence of the salt gland is shown in blue, the cellular autofluorescence in green and the reflected light in gray.**

## The “waffle” workflow for extracted *Arabidopsis thaliana* embryos

*A. thaliana* ecotype Columbia (Col\_0) was grown 5-7 weeks in soil at 22.5° with a photoperiod of 16/8 h (day/night). The isolation of embryos was performed on the preparation table of the Leica EM-ICE high-pressure freezer using its integrated binocular microscope. As freezing buffer, 20% Ficoll 400 (w/v) dissolved in PBS was used. The first eight siliques were cut with fine scissors and placed on a glass slide in a drop of freezing buffer. Next, embryos were hand dissected with sharp tweezers as described in reference<sup>40</sup> and collected. A 50 mesh EM-grid with continuous support film (Carbon coated Formvar, Graticules Optics Ltd, Tonbridge, UK) was placed with the support facing down in a 3 µm droplet of freezing buffer on the flat side of a cetyl palmitate coated 6 mm Type B HPF carrier. The excess of buffer was blotted away with filter paper until the support film was flat on the carrier. Several embryos were picked up with tweezers and placed in the wells formed by grid bars and support film (Extended Data Fig. 10d). A spacer ring (Provider) with a height of 50 µm was added onto the grid and filled with a 2 µL drop of freezing buffer. Air bubbles were removed with tweezers. Afterwards, the sandwich was closed with a second 6 mm type B carrier flat side facing down, the sample high-pressure frozen, the grid recovered and stored in a grid box in LN<sub>2</sub>.

## Targeting the salt gland of in-carrier frozen *Limonium bicolor*

The culture conditions *L. bicolor* Kuntze, fluorescence microscopy detection of salt glands and HPF procedures are similar to those described previously<sup>41</sup> with minor modifications. Namely, *L. bicolor* seeds were stored in a refrigerator at 4 C before use. The seeds were then surface disinfected in 70% (v/v) ethanol for 5 min and subsequently in 6% (w/v) sodium hypochlorite (freshly prepared) for 20 min and then thoroughly washed with deionized water. Fully filled seeds were selected and planted in MS culture medium plates. These plates were incubated in a plant

tissue culture room at a temperature of 26/22°C (day/night), a photoperiod of 16/8 h (day/night) and a light intensity of 600  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . After 2 weeks, the first true leaf (FL, Extended Data Fig. 10f) of the whole plant was dissected with a thin knife, placed in the 300  $\mu\text{m}$  deep cavity of a 3 mm Type B HPF carrier filled with 20% (w/v) Ficoll 400. Afterwards, the cavity was closed off with a second Type B carrier, flat side facing down, the specimen was high-pressure frozen and the sample containing carrier recovered. Fluorescence targeting of the salt gland was performed using the int-FLM recording green autofluorescence (excitation wavelength = 485 nm, emission wavelength = 525 nm), blue autofluorescence (excitation wavelength = 385 nm, emission wavelength = 432 nm) as well as the reflected light (excitation wavelength = 385 nm, no emission filter, Extended Data Fig. 10g). Serial Lift-Out with the attachment from below was then performed as described in the previous sections (Extended Data Fig. 10h).

## TEM Imaging

One part of the TEM data was collected on a Titan Krios G2 (Thermo Fisher Scientific) with BioQuantum energy filter (Gatan, Pleasanton, California, USA) and K2 summit camera (Gatan). Automated data acquisition was performed in SerialEM<sup>42,43</sup> (version 4.1). Lamella montages were recorded at 8700x magnification (pixel size = 2.6 nm). Tomograms were acquired at 42000x, 53000x or 64000x magnification (pixel sizes of 3.52 Å, 2.62 Å and 1.98 Å, respectively), at target defoci of -4  $\mu\text{m}$  to -6  $\mu\text{m}$ , using a dose symmetric tilt scheme between  $\pm 50^\circ$  to  $\pm 60^\circ$  with an angular increment of  $2^\circ$  or  $3^\circ$ , taking the pretilt of the lamellae into account. The exposure time was kept constant for all tilt images resulting in total doses per tilt-series of 80-125  $\text{e}^-/\text{\AA}^2$ . Typically, 10 frames were recorded per tilt image and saved in TIF-file format.

The other part of the data was recorded on a Titan Krios G4 equipped with a Selectris X energy filter and a Falcon 4i camera (Thermo Fisher Scientific). Lamella montages were recorded at a

magnification of 8700x (pixel size = 2.8 nm). Tomograms were recorded at 42000x or 64000x (pixel sizes of 2.93 Å and 1.89 Å, respectively) with defoci between -3 µm and -6 µm. Data was acquired using the Tomography 5 software package version 5.12.0 (Thermo Fisher Scientific) and stored in EER file format.

## Data preprocessing

Tilt-series were processed using the TOMOMAN<sup>44</sup> (version 0.6.9) pipeline. Movie frames were motion corrected in MotionCor2<sup>45</sup> (version 1.4.7). EER frames were grouped to obtain between 8 and 20 frames per movie. Bad tilt images, i.e. improperly tracked images or blurry images due to lamella instabilities, were removed after manual inspection and dose-weighting was performed according to<sup>46</sup> using the corresponding TOMOMAN scripts. If tilt-series contained at least 5 platinum fiducials introduced by sputtering evenly distributed over the field-of-view, IMOD<sup>47</sup> (version 4.11.25) fiducial tracking was employed for tilt-series alignment. Otherwise, AreTomo<sup>48</sup> version 1.3.3 was used for tilt-series alignment. Afterwards, 4x binned tomograms were reconstructed with the weighted back projection algorithm implemented in IMOD. For denoising, tilt series containing only the information of odd or even frames were obtained during motion correction. The odd and even tilt-series were preprocessed to generate 4x binned odd and even tomograms using the same alignment files as for the full tilt-series. Denoising was performed in cryo-CARE<sup>49</sup>. First, 800-1000 training volumes with a box size 72<sup>3</sup> voxel were cropped from the odd and even tomograms. A network was trained for each tomogram over 150 epochs with 75 steps and a Unet depth of 3. The network was then used to denoise the 4x binned tomograms. All TEM data, including tomograms and lamella overviews, were visualized with IMOD<sup>50</sup> (version 4.11.25). Only slices of denoised tomograms are shown in this work.

## Subtomogram averaging of rubisco

The detailed workflow for subtomogram averaging and spatial analysis for rubisco is described in the Supplementary Information. In brief, a subset of 10 tomograms were recorded on *P. patens* protonemata chloroplasts with defoci between -2.5 and -5  $\mu\text{m}$ . Tilt-series were aligned and reconstructed in IMOD and imported into WARP<sup>51</sup> (version 1.0.9). Membranes were segmented using MemBrain-seg<sup>52</sup> and the chloroplast volumes were manually annotated in Amira (Amira 3D 2021.2, Thermo Fisher Scientific) to create a mask for score maps as output of template matching. A first template was created by picking 500 rubisco complexes manually (50 complexes per tomogram) and aligning them in RELION<sup>53</sup> (version 3.0.5) rubisco was picked by template matching in StopGap<sup>54</sup> (version 0.7.0). After several rounds of subtomogram alignment and classification in RELION and tilt-series alignment in M<sup>55</sup> (version 1.0.9) the final density map was calculated using 6,180 particles and applying C4 symmetry (Fig. 4c). An octamer of large rubisco subunits (monomer UniProt accession P34915) was predicted in AlphaFold3<sup>28</sup> and docked into the final subtomogram average using the “fit” function in ChimeraX<sup>56</sup> (version 1.6.1) without further refinement of the model (Fig. 4c-d). The spatial analysis of rubisco particles was performed based on 55,000 particles after a first round of classification in RELION similar to<sup>57</sup>. The 3D rendered image of chloroplast membranes and rubisco positions was displayed in ChimeraX using the ArtiaX plug-in<sup>58</sup>.

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