

1 Automated cryo-lamella preparation for high-throughput *in-situ* 2 structural biology

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14

15 Keywords:

16 Cryo-FIB, cryo-lamella, automation, cryo-EM, in situ structural biology

17 Abstract

18 Cryo-transmission electron tomography (cryo-ET) in association with cryo-focused ion beam (cryo-FIB)
19 milling enables structural biology studies to be performed directly within the cellular environment.
20 Cryo-preserved cells are milled and a lamella with a thickness of 200-300 nm provides an electron
21 transparent window suitable for cryo-ET imaging. Cryo-FIB milling is an effective method, but it is a
22 tedious and time-consuming process, which typically results in ~10 lamellae per day. Here, we
23 introduce an automated method to reproducibly prepare cryo-lamellae on a grid and reduce the
24 amount of human supervision. We tested the routine on cryo-preserved *Saccharomyces cerevisiae*
25 and demonstrate that this method allows an increased throughput, achieving a rate of 5 lamellae/hour
26 without the need to supervise the FIB milling. We demonstrate that the quality of the lamellae is
27 consistent throughout the preparation and their compatibility with cryo-ET analyses.

28

29 Introduction

30 Today, the best method to image the cellular environment in its native state and obtain structural
31 information is Cryo-Electron Tomography (cryo-ET) (Beck & Baumeister, 2016). Biological samples are
32 vitrified by fast freezing at cryogenic temperature (80 – 120 Kelvin) and then imaged using a cryo-
33 Transmission Electron Microscope (TEM) following a tomographic acquisition scheme. Cryogenic
34 fixation is instantaneous and it helps to reduce the effects of radiation damage during data acquisition
35 (Dubochet et al., 1988). This process ensures that all the water contained in the sample is vitreous,
36 therefore solid and capable of withstanding the high-vacuum environment of TEMs while not
37 diffracting the electron beam. As a general guideline, the maximum allowed sample thickness is
38 ~300nm, since most cryo-TEM operate at 300 keV where an electron statistically undergoes a single
39 elastic scattering event in ~250 nm of water (elastic mean free path) (Holtz, Yu, Gao, Abruña, & Muller,
40 2013). Thicker samples provide higher chances of inelastic scattering, which in non-crystalline samples
41 cannot be interpreted as useable information. Since cells are typically multiple microns thick, the
42 inspection is limited to the thinnest regions such as filopodia (Carlson et al., 2010; Stauffer et al., 2014;
43 Weber, Wojtynek, & Medalia, 2019). The first approach that made it possible to inspect the inside of
44 a cryo-preserved cell through cryo-ET was cryo-sectioning of vitreous sections (CEMOVIS) (Al-Amoudi
45 et al., 2004). CEMOVIS has the advantage of potentially allowing serial-section tomography with
46 minimal losses throughout the cell, but it is extremely difficult to perform and suffers from mechanical
47 deformations and is prone to surface contamination (Al-Amoudi, Studer, & Dubochet, 2005).
48 Alternatively, using an extremely common approach in semi-conductor sciences, it is possible to
49 isolate a thin lamella of material using a dual-beam microscope which utilizes a Scanning Electron
50 Microscope (SEM) for imaging and a Focussed Ion Beam (FIB) for machining any sample with
51 nanometre resolution (Marko, Hsieh, Schalek, Frank, & Mannella, 2007; Rigort et al., 2012; Schaffer
52 et al., 2015; Schaffer et al., 2017). Cryo-FIB milling has been shown to be a highly effective method for
53 preparing cellular sections for cryo-electron tomography (Rigort et al., 2012; Schaffer et al., 2015;
54 Schaffer et al., 2017; Villa, Schaffer, Plitzko, & Baumeister, 2013), but it is time-consuming since the
55 resolution and the milling speed are inversely proportional.

56 In contrast to industrial FIB applications (e.g. semi-conductors), all existing procedures for cryo-
57 lamellae preparation are performed manually due to the heterogeneity and dose-sensitivity of
58 biological specimens. The immediate effect is that highly trained users are performing an extremely
59 repetitive task. This not only results in an elevated cost, but it also restricts production to a throughput
60 of 5-10 lamellae per day (Danev, Yanagisawa, & Kikkawa, 2019; Koning, Koster, & Sharp, 2018; Wolff
61 et al., 2019). Further, in most cases, cryo-FIB microscopes are only used for one session/day while
62 users are onsite, therefore to boost the productivity night shifts are the only way forward. Cryo-ET

63 acquisition can already run unsupervised and regardless of the acquisition scheme used it is possible
64 to obtain at least 20 tomograms over 24 hours. Data collection schemes optimized for high resolution
65 will require ~60 min of acquisition time per tomogram (Hagen, Wan, & Briggs, 2017), while fast
66 tomography workflows will allow for acquisition times down to 5 minutes (Chreifi, Chen, Metskas,
67 Kaplan, & Jensen, 2019; Eisenstein, Danev, & Pilhofer, 2019). Moving beyond the bare throughput and
68 instrument usage, since FIB milling is a manual and iterative process the reproducibility scales with
69 the user experience. Recognising the need for reproducible sample preparation, several workflows
70 and best practice procedures have been developed (Hsieh, Schmelzer, Kishchenko, Wagenknecht, &
71 Marko, 2014; Medeiros et al., 2018; Schaffer et al., 2015), but the initial steep learning curve still
72 represent a limitation especially for laboratories where in house expertise has not yet been
73 established.

74 One way to counteract the low throughput of cryo-lamella preparation consists of targeting a region
75 based on the known presence of an interesting event or structure. In order to achieve this level of
76 efficiency, the typical approach uses correlative light and electron microscopy techniques which, when
77 properly performed, prevents from imaging areas that do not contain useful information for a specific
78 study (Arnold et al., 2016; Gorelick et al., 2019). Regardless of the correlative microscopy approach in
79 use, the mismatch between the throughput of cryo-ET and the preparation of lamellae is evident. Here
80 we introduce an automated and reproducible method for on-grid cryo-lamella preparation for vitrified
81 cell. We demonstrate an increased throughput capable of reproducibly producing ~5 lamellae/hour.
82 Since the presented protocol can run unsupervised, the learning phase for new users will be shorter
83 than the current situation. Further, with the appropriate cryo-stage, this protocol opens the possibility
84 for a 24/7 cryo-lamellae preparation.

85 Materials and methods

86 Cell culture and sample cryo-fixation

87 *Saccharomyces cerevisiae* was cultured in YPD at 30 degrees, cells were harvested when an optical
88 density of 0.6 was reached. Cells were washed twice in PBS and re-suspended in half of the initial
89 volume. 5 µL of cell-containing solution was deposited on a Cu-300 R2/2 grid (Quantifoil™) which had
90 been previously glow discharged for 30 seconds using a Pelco EasyGlow™. Excess solution was
91 removed through manual blotting and cells were vitrified by plunge freezing in a liquid 60/40
92 Ethane/propane mix and stored in liquid nitrogen.

93 Cryo-FIB milling

94 All tests presented here were conducted on a ThermoFisher Helios Ux G4 DualBeam™ equipped with
95 a Leica cryo-stage and a VCT500 cryo-transfer system. Grids were clipped in autogrid cartridges prior
96 to loading into the cryo-FIB.

97 Cryo-FIB control and scripting

98 All manual operations were conducted through the ThermoFisher Xt UI™, while batch cryo-FIB
99 operations were performed using Python 3.6 through the communication API ThermoFisher
100 Autoscript 4.1™ (which is required to control the microscope). Scientific python packages used include
101 numpy, scipy, matplotlib, scikit-image, click, pyyaml and pytest (Hunter, 2007; Oliphant, 2007; S. van
102 der Walt, Colbert, & Varoquaux, 2011; Stéfan van der Walt et al., 2014). All original scripts used in this
103 procedure are installable via the pip package installer for Python. All the source code and the
104 instructions can be downloaded from the laboratory webpage
105 (www.github.com/DeMarcoLab/autolamella).

106 Cryo-TEM

107 Cryo-electron tomograms and micrographs were collected on a ThermoFisher Titan Krios G3 equipped
108 with a post-GIF Gatan K2™ DDE camera. Images were collected in EFTEM mode following a low-dose
109 acquisition scheme. The microscope setup included a 50 µm condenser aperture and a 70 µm
110 objective aperture. All tomograms were imaged from -60 to +60 deg with 3 deg increment. The total
111 dose on the tilt-series was 98.4 e⁻/Å², with a dose-rate of 4e⁻/pixel/sec and tilt micrographs were
112 collected at a nominal magnification of 42000x resulting in a final pixel-size of 3.61 Å. Low
113 magnification overviews were collected at a nominal magnification of 2500x in LM mode, resulting in
114 a final pixel-size of 20 nm.

115 Tomography reconstruction

116 Tilt-series were aligned using patch tracking and back-projected using Etomo from the IMOD suite
117 (Mastronarde, 1997). Tomogram visualization and filtering occurred through IMOD or UCSF Chimera
118 (Pettersen et al., 2004).

119 Image display and figure preparation

120 All figures have been prepared using Adobe Illustrator. Cropping, filtering and contrast adjustments
121 have been performed using IMOD or Fiji. Videos have been made using Fiji (Schindelin et al., 2012).

123 Results and discussion

124 Any TEM sample preparation procedure based on FIB milling requires the following operations:
125 changing the milling current and/or energy, changing the imaging parameters, stage movements for
126 sample realignment, beam-shift for drift compensation and setup/positioning of the milling patterns.
127 On average, every milling step can require time spanning from a few seconds to multiple minutes and
128 the sequence is extremely repetitive and predictable. In this situation, even the most diligent operator

129 will incur a significant time-waste as a consequence of short delays at the start of every step and due
130 to the time required to perform manual realignment.

131 Here, we analysed the preparation workflow and reduced its complexity to the 4 steps: (i)
132 identification of the regions of interest; (ii) opening the lamellae (trenching); (iii) thinning and (iv)
133 polishing the lamellae. Cryo-FIB milling occurs in a high-vacuum environment where the pressure
134 ranges from $2e-6$ to $5e-7$ mBar. Under these conditions the atmosphere around the sample is not
135 completely depleted from water, therefore, the slow growth of a crystalline ice layer on the sample
136 surface is expected over time. Typical contaminations rates measured as crystalline water deposition
137 are specified by the instrument manufacturers to be in the order of 5 – 20 nm/hour. In addition, the
138 material that is sputtered while milling can redeposit on neighbouring areas, therefore contaminating
139 finished lamellae. To limit contamination, we concluded that the best course of action is to run the
140 lamella preparation protocol in 3 or more stages. Accordingly, we defined a default protocol that
141 includes 3 stages, first the routine loops through all the regions of interest and open the trenches,
142 then lamellae are thinned to ~ 500 nm and last they are polished to the desired thickness (100-300
143 nm). Following this protocol, the bulk material removal, which also represents the most time-
144 consuming part is carried out over the first two steps, while the polishing step is performed over the
145 last few minutes prior to unloading the sample. The default number of stages for the preparation of
146 the lamellae is three, but users can add or remove stages if the number of conditions required to
147 achieve good lamellae is different on other samples. The presented routine includes stage movement,
148 beam current changes and, most importantly, it requires time. Accordingly, there will be drift and
149 relocation errors to be accounted for. Our proposed protocol follows a low-dose approach similar to
150 the one performed in cryo-ET, where a sacrificial area containing a fiducial marker is used for tracking
151 and focussing, whereas the sample area is imaged only once at low-dose and magnification to define
152 the location of the region of interest.

153 Figure 1 shows a flowchart diagram of the program logic. We minimized the required user interaction:
154 the user provides a configuration file when launching the program (an example file is provided with
155 the package) which contains all the conditions required for the lamella preparation. The parameters
156 defined in the configuration file are fixed for each batch job and include the milling and imaging
157 currents, the depths, the field of view size, as well as the fiducial and the lamella sizes. The milling
158 depth for lamellae and fiducials are set in the configuration file, but, where required, they can be set
159 independently for each location. This decision was made to provide a good compromise between the
160 flexibility and setup time. Lamellae locations are interactively selected together with the location of
161 the tracking area. In order to improve reproducibility, a small cross-shaped fiducial marker is FIB milled
162 in the centre of the tracking area. The fiducial marker assists image re-alignment after stage

163 movements and shifts due to changes in the ion beam current. The image containing the fiducial
164 marker is updated after every re-alignment to take care of eventual degradations that might occur in
165 response to imaging. In principle, this area can be used to correct against drift during the milling
166 procedure, but we found that on two systems with different cryo-stages (both ThermoFisher Helios
167 G4) this option is not needed given the short time that each milling stage requires. Since the sample
168 thickness can vary substantially across locations, we kept the possibility to adjust the desired FIB
169 milling depth individually for each lamella and fiducial. This setup procedure is repeated for as many
170 locations as the user wishes to mill and the only constraint here is the time required for milling. The
171 user is guided step-by-step throughout the setup phase (for examples through the workflow see
172 supplementary figures 3-6). The tracking area is also used to re-align the lamella position when
173 compensating for the beam convergence (Schaffer et al., 2017). For this option, users are able to
174 define the amount of over/under-tilt to apply in relation to the current and instrument in use.
175 Since the tracking procedure follows a low-dose acquisition scheme, the site where the lamella will be
176 prepared is going to be imaged only once using the ion beam and twice using the SEM (optional). In
177 order to help the users during the protocol optimization for each sample and for documentation
178 purposes, we added the option to acquire full-field ion beam images at each stage of the process, but
179 in general, this option would not be used so the sample conditions can remain pristine. The ion beam
180 image that is acquired at the beginning of the process to define the lamella position and the tracking
181 area generally will have a low resolution and fast dwell time to minimize the dose applied to the
182 sample. In our experiments, we have found that a field of view of 50 μm acquired with a pixel size of
183 32nm provides a good image quality when using 10-30 pA of current with dwell times comprised
184 between 50 and 300 nsec. Under these conditions, the dose is extremely low but the alignment
185 precision is limited by the pixel size. Accordingly, we have set the possibility of acquiring the tracking
186 images (restricted to the sacrificial area) with a smaller pixel size (e.g. 8 or 16 nm) and dose, therefore
187 making the alignment process significantly more precise. Further, in order to increase the flexibility in
188 case a user needs to image samples that do not align efficiently with the provided algorithm, we set
189 up the code structure to allow custom frequency filtering and real space masks to be applied, or even
190 the replacement of the alignment function with another algorithm.
191 In figure 2 we show a gallery through the lamella preparation procedure. The cross mark visible in the
192 image represents the centre of the tracking area and has a size of 6 \times 6 μm . In order to respond to the
193 variability of the sample topology, we made sure that the position of the fiducial and the lamella can
194 be selected and do not have a strict relative position. The only limits set are to prevent the milling
195 areas to overlap between fiducial and lamella as well as to prevent any of the two areas to be outside
196 the field of view.

197 We tested the robustness of the procedure by setting up batch preparation jobs with multiple (3-5)
198 lamellae. Our results indicate high reproducibility throughout each job with lamellae thicknesses
199 always comprised between 210 nm and 250 nm. The throughput was measured to be 4 to 5
200 lamellae/hour depending on the sample thickness. In supplementary figure 1, we show a gallery
201 comprising 5 lamellae which have been prepared in 47 minutes from the beginning of the batch
202 procedure. The setup phase (selecting sites and defining tracking markers) for that job required
203 approximately 12 minutes. In order to ensure that the quality of the samples is not compromised, we
204 inspected all lamellae through cryo-TEM (see figure 3 and supplementary figure 2), and imaged a
205 subset via cryo-ET (figure 4 and supplementary video), therefore ensuring the suitability for
206 downstream analyses. The level of curtaining is comparable to what has previously been reported
207 from manual preparation. Although in this case, we have noticed that the length of the lamella directly
208 correlates with the level of curtaining. This observation correlates with the fact that we applied the
209 same ion dose when preparing all lamellae (supplementary figure 2).

210 Our results show that by automating the lamella preparation it is possible to prepare uniform lamellae
211 with the desired thickness at a rate which is far greater than what could be reproducibly achieved with
212 manual operation. The average preparation time for 1 lamella is ~9 minutes, excluding sample loading,
213 initial alignment and selection of the regions of interest. From experience, users will require about 1
214 hour for stage cooling and sample loading, then Pt deposition and general imaging of the samples for
215 navigation will require ~10-15 min. Then, when the procedure starts, the basic navigation is conducted
216 through the microscope UI and the user must ensure that when a region of interest is selected the
217 sample is at the coincidence height. This is important because the overview images acquired with the
218 SEM before and at the end of the milling procedure can provide immediate feedback on the quality of
219 each lamella before going to the cryo-TEM. If the stage is not at the correct height the SEM images
220 will not include the lamella site. Further, if the user chooses to compensate for the beam convergence
221 (optional), the stage tilt will change by 1-4 deg. If the stage is not at the correct height the region of
222 interest will move outside the field of view. Our experience shows that the user supervised part of the
223 procedure which includes the selection of the regions of interest and the preparation of the fiducial
224 requires ~2-3 minutes/site. Accordingly, if a user loads 2 grids and selects 20 sites/grid the preparation
225 time from stage cooling to the completion of 40 lamellae would be between 10 and 11 h, with the
226 polishing step conducted through the last 1.5 h.

227 The typical duration of a cryo-FIB session is limited by the liquid nitrogen supply (ThermoFisher and
228 Quorum cryo-stages can be kept cold for ~11 h, while Leica cryo-stages have the possibility to refill
229 the liquid nitrogen dewar and provide sessions that are longer than 24 h). The automation and the
230 fact that the procedure runs mostly unsupervised ensures that for ~8 h users do not need to be on-

231 site. Accordingly, this opens up the possibility to prepare cryo-lamellae with a 24 h continuous
232 operation of the microscope and therefore lead to ~80 or more lamellae/day. Considering the latest
233 developments in regards to fast cryo-ET acquisition it will be possible to match this throughput on the
234 TEM (Chreifi et al., 2019). Even in the situation where the above-described throughput is not required,
235 a protocol providing up to 40 lamellae during a daytime session will shorten the user queue around
236 cryo-FIBs, therefore, allowing more projects to be conducted in facilities where the availability of
237 instrumentation is limited in relation to the number of users.

238

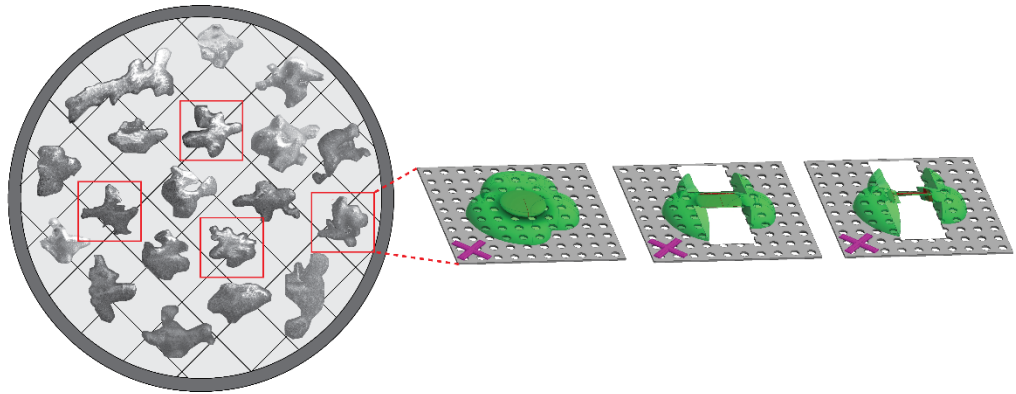
239

240 Acknowledgements

241 We acknowledge the support and expertise of the Clive and Vera Ramaciotti Platform for Structural
242 Cryo-Electron Microscopy (Monash University, Clayton). GB, CT, JCW and AdM acknowledge the
243 support of an Australian Research Council Laureate Fellowship and the ARC Centre of Excellence in
244 Advanced Molecular Imaging. JCW further acknowledges the support of the National Health and
245 Medical Research Council of Australia Senior Principal Research Fellowship and Program grant. We
246 also thank Sergey Gorelick for his contribution to the graphical abstract.

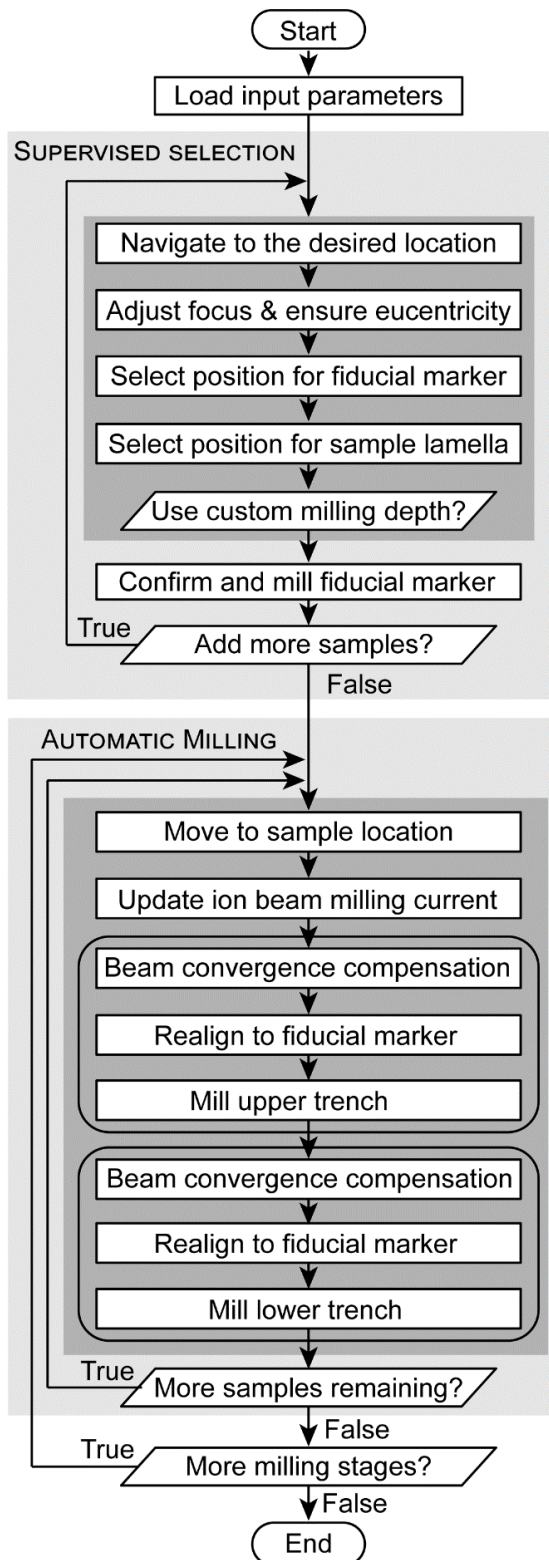
247

248 Figures



249

250 **Graphical abstract.**



251

252 **Figure 1: Flowchart of the automated cryo-lamella process.** First, a configuration file containing all

253 the required parameters is loaded (e.g. lamella size, currents, etc.). The process can be divided into

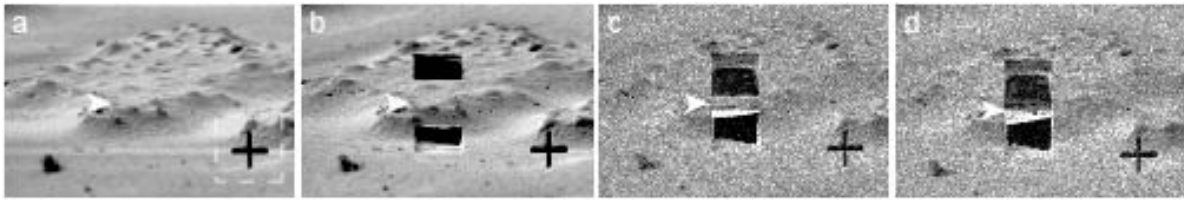
254 two sections, a human supervised section followed by the automated lamella preparation across all

255 selected locations. The supervised section requires users to interactively select the locations. The

256 general navigation can be performed through the ThermoFisher Xt UI™ or ThermoFisher MAPS™

257 (where available). In order to achieve an optimal performance, the user must adjust the focus and
258 ensure the sample stage is at the coincidence point between the FIB and SEM beams. The position of
259 the fiducial marker (used for image realignment) and the lamella can be defined anywhere within the
260 field of view. This section is performed as many times as the number of lamellae required. Once all
261 sites of interest have been selected the automated section starts. Here, automated ion beam milling
262 is performed for all selected samples. First, the initial trenches are opened for every sample (typically
263 using a higher milling current). Then the FIB milling current is reduced and every sample is further
264 thinned until the final polishing step. Every time the sample stage is moved or the FIB milling current
265 is changed, the sample position is checked using the fiducial marker and correction is done through
266 beam-shift. The compensation for the beam convergence can be specified in the input configuration
267 file and can be changed for each milling stage.

268

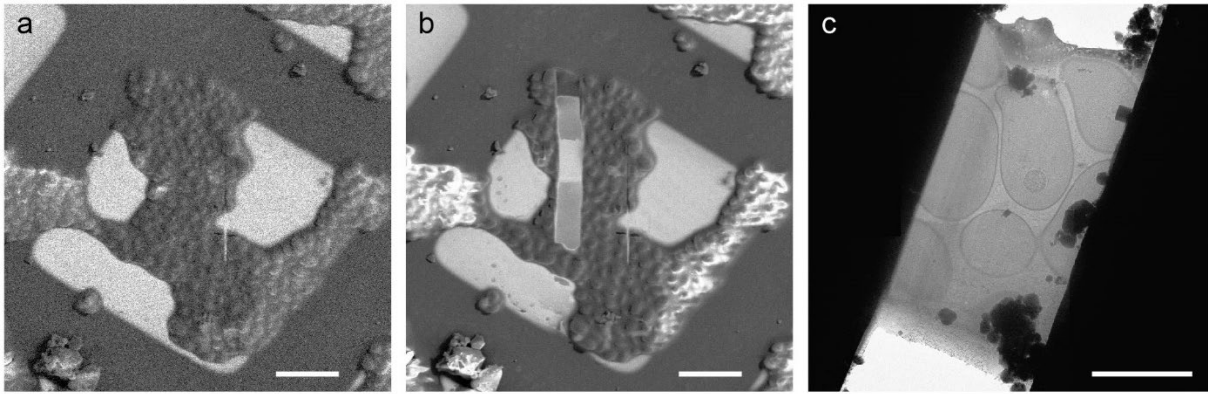


269

270 **Figure 2: Ion beam images of a single lamella during the automated FIB milling.** (a) A cross-shaped
271 fiducial marker is created for later image realignment, reduced area field of view used for realignment
272 shown by dashed white box; (b) trenches are milled above and below the lamella using a current of
273 2.4 nA; (c) the lamella is thinned further using an intermediate ion beam current, 75 pA; (d) the
274 completed lamella after final ion beam milling at low current, 26 pA. White arrowheads indicate the
275 position of the finished lamella; the scale is indicated by 6×6 μm cross-shaped fiducial marker.

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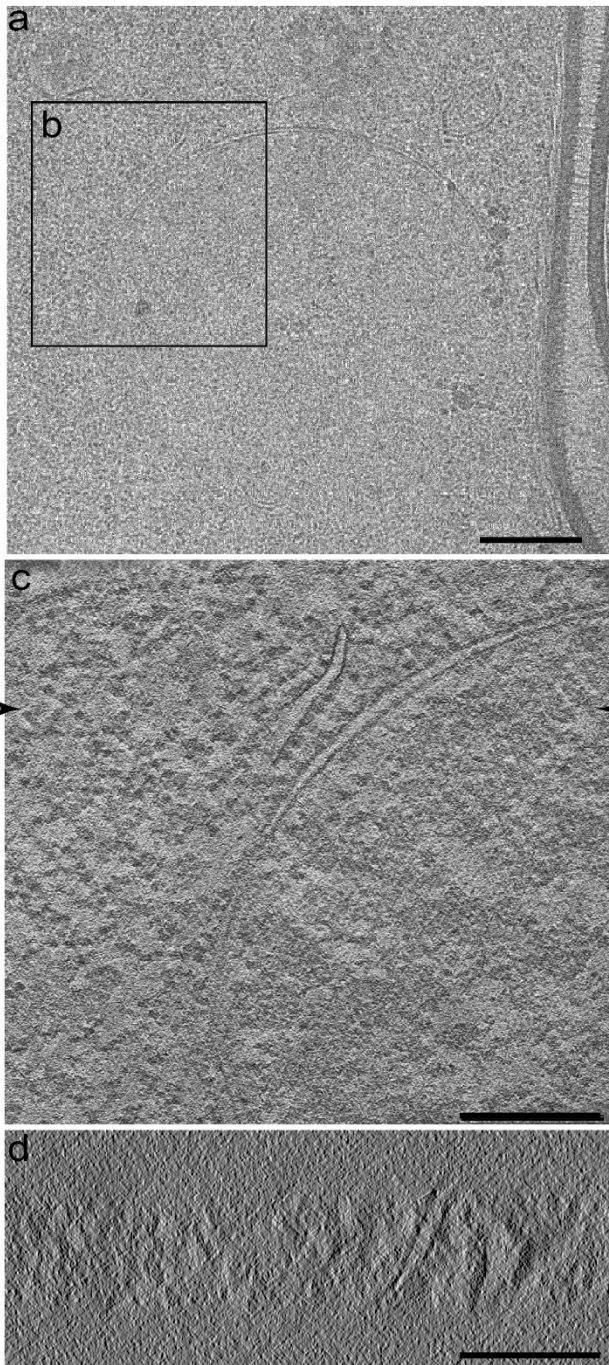
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Figure 3: View of an exemplary lamella from cryo-SEM and low magnification cryo-TEM. (a) shows a cryo-SEM view of a region before the lamella preparation; (b) shows the same region after sample preparation. Both SEM images have been acquired as part of the automated procedure. (c) shows a low magnification cryo-TEM overview of the same lamella displayed in a and b. The surface quality is comparable to what has previously been reported from manual preparation. Scale-bars in (a) and (b) are 20 μm , while in (c) the scale bar 5 μm .

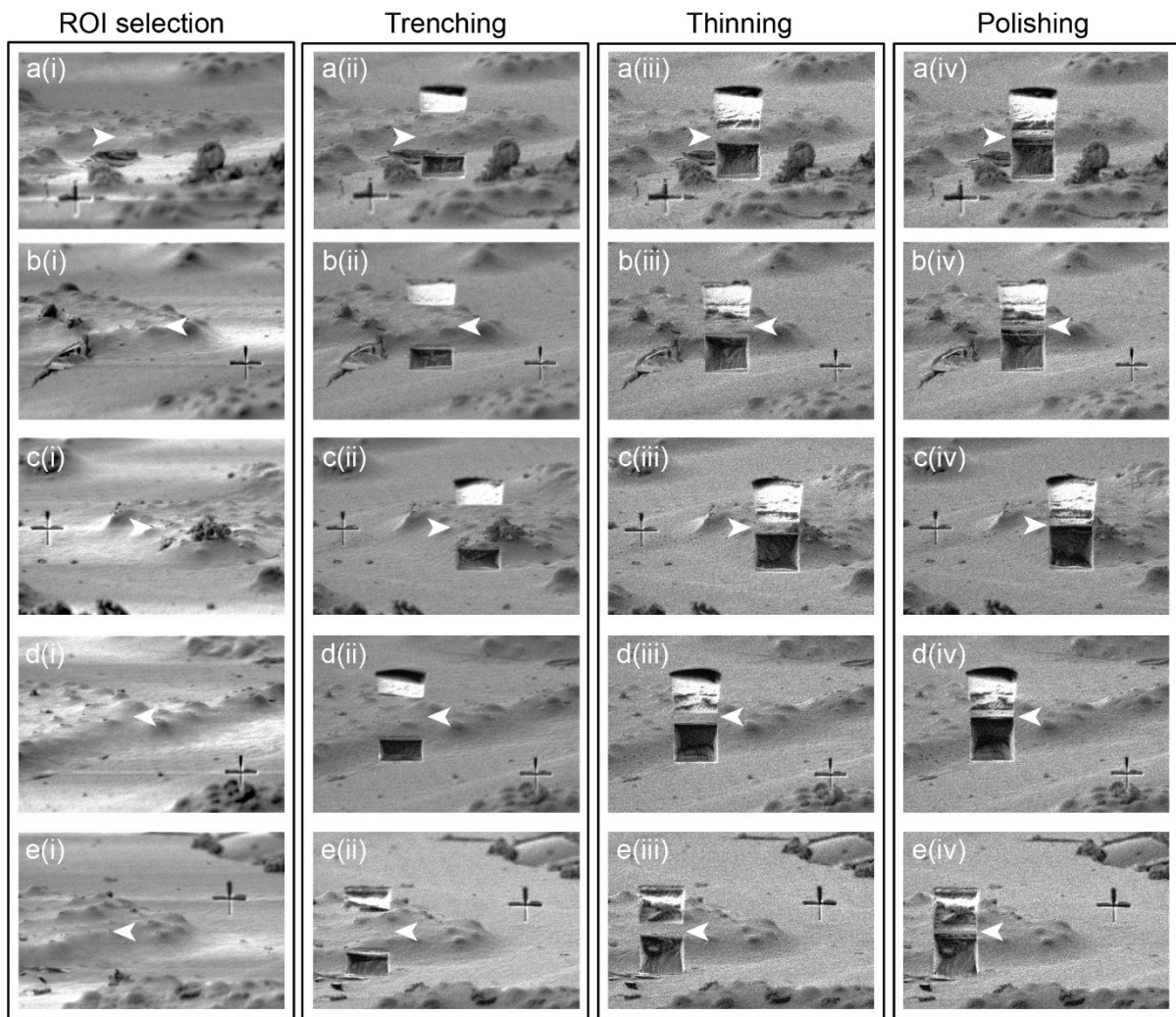


287

288 **Figure 4: Cryo-TEM and cryo-ET performed on the same lamella shown in figure 2. Panel (a) shows a**
289 **mid-magnification (6500x) micrograph of the lamella. Inset (b) in panel (a) corresponds to the XY slice**
290 **from the tomogram collected and displayed in (c). Panel (d) shows a XZ slice through the tomogram.**
291 **The arrowheads in (c) show the position of the slice in displayed in (d). Scale-bars are 500 nm in (a)**
292 **and 200 nm in (c) and (d).**

293

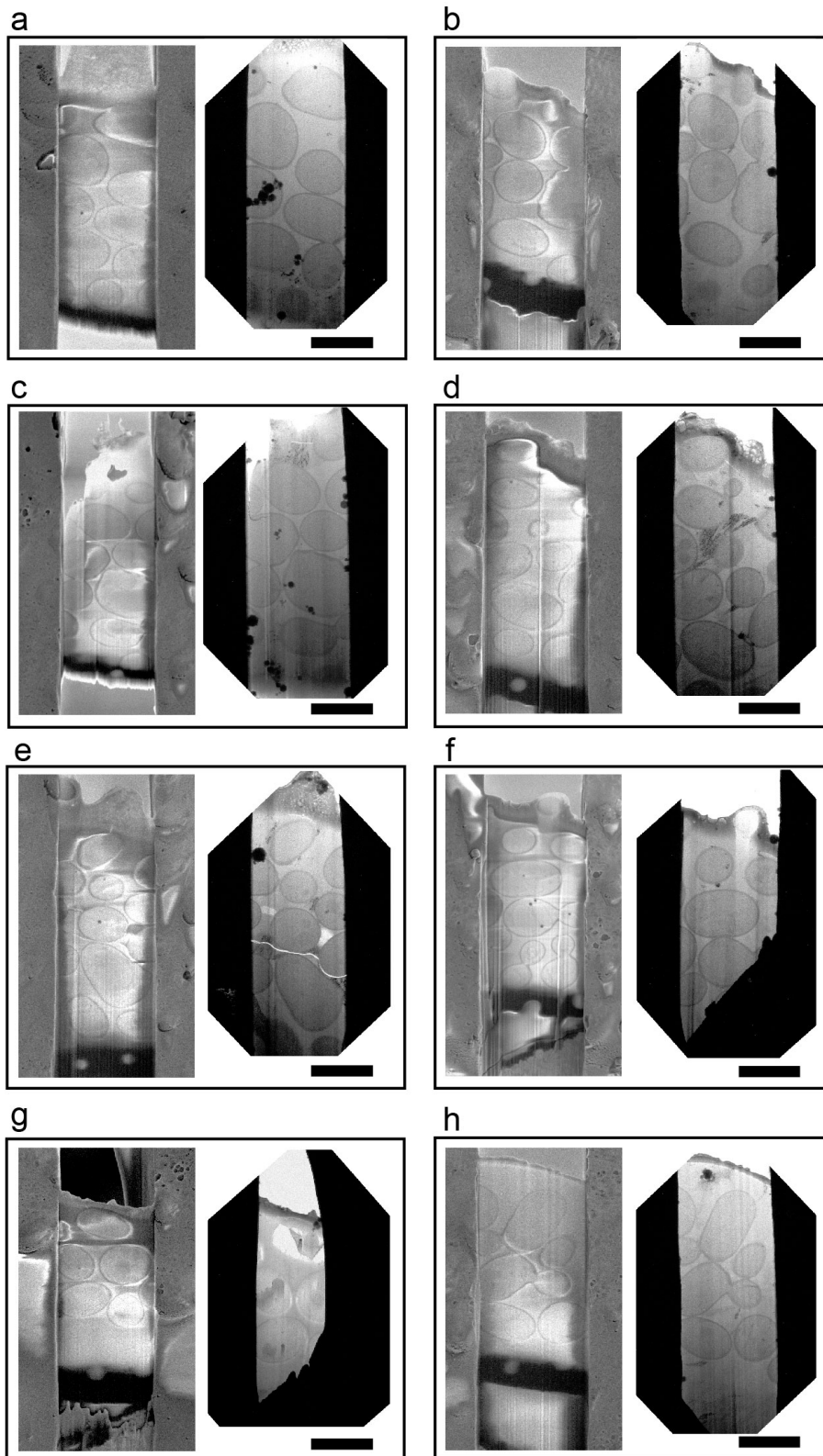
294 Supplementary Information



295

296 **Supplementary figure 1: Ion beam images acquired throughout a single batch job consisting of 5**
297 **lamellae.** The total duration of the batch job was 47 minutes, plus 12 minutes required for the initial
298 (manual) selection of sample locations by the user. Here, each lamella location is displayed in a single
299 row (marked a, b, c, d, e) and white arrowheads indicate the position of the finished lamella. The cross-
300 shaped fiducial marker acts as scale-bar, with dimensions of $6 \times 6 \mu\text{m}$. Columns from left to right show:
301 (i) the fiducial marker is created; (ii) trenches opened with high ion beam current; (iii) further thinning
302 with an intermediate ion beam current and (iv) the completed lamella after final polishing with low
303 ion beam current. Ion beam milling currents for these stages were 2.4 nA, 75 pA, and 26 pA,
304 respectively. The milling procedure runs per column, therefore, each FIB milling stage is completed
305 for every lamella location before proceeding to the next milling stage.

306



307

308 **Supplementary figure 2: Gallery of 8 lamellae prepared using the procedure described in this article,**

309 **illustrating quality and pitfalls.** Each panel shows on the left a low voltage cryo-SEM image of the

310 lamella surface at the end of the preparation procedure. On the right within each panel there is a low

311 mag cryo-TEM micrograph for each lamella. The images show that the quality is comparable to current

312 preparation approaches. In this gallery, we show potential pitfalls of batch preparation which can be

313 easily resolved if the user is aware and careful. The level of curtaining changes in response to lamella
314 length and surface contamination, if the sample thickness is variable the user should select more
315 generous depth for the polishing step to ensure the surface finish is optimal. (a, b) show proper depth
316 selection, while (c–h) show different behaviours when under-polishing). Panels (f) and (g) show that
317 the size of the trenches was not appropriate for the sample thickness, as visible from the cryo-TEM
318 images part of those lamellae is not electron transparent, indicating that there is bulk material
319 underneath the lamella (also visible from the cryo-SEM). Scale-bars are 5 μm .
320

Select Anaconda Prompt - autolamella config.yml

```
(base) C:\Users\genevieb>conda activate fibsem
(fibsem) C:\Users\genevieb>autolamella config.yml
Create a new EMPTY directory to store your output images.
Client connecting to [localhost:7520]...
Client connected to [localhost:7520]
2019-09-17 15:52:57,754 INFO demo_mode: 1.0
fiducial:
  fiducial_image_size_x: 1.5e-06
  fiducial_image_size_y: 1.5e-06
  fiducial_length: 5.0e-07
  fiducial_milling_current: 3.0e-10
  fiducial_milling_depth: 1.0e-07
  fiducial_width: 1.0e-07
  min_distance_from_lamella: 1.0e-07
  reduced_area_resolution: 3072x2048
imaging:
  autocontrast: 0.0
  dwell_time: 1.0e-07
  full_field_ib_images: 1.0
  horizontal_field_width: 5.0e-05
  resolution: 1536x1024
lamella:
  lamella_height: 1.0e-07
  lamella_width: 5.0e-07
  milling_current: 3.0e-10
  milling_depth: 1.0e-06
  overtilt_degrees: 0.0
  protocol_stages:
  - milling_current: 3.0e-10
    milling_depth: 5.0e-07
    percentage_from_lamella_surface: 0.5
    percentage_roi_height: 0.5
  - milling_current: 3.0e-10
    percentage_from_lamella_surface: 0.2
    percentage_roi_height: 0.3
  - milling_current: 3.0e-10
    overtilt_degrees: 2.0
    percentage_from_lamella_surface: 0.0
    percentage_roi_height: 0.2
  total_cut_height: 5.0e-07
save_directory: C:/Users/genevieb/Documents/test/output_directory
system:
  application_file_cleaning_cross_section: Si
  application_file_rectangle: Si
  ip_address: localhost
```

321

322 **Supplementary figure 3: Screenshot of the terminal as the user launches the ‘autolamella’ program.**

323 The first section of the output consists of a summary of the parameters provided in the configuration

324 file, here named config.yml. The idea is to provide immediate feedback that the file and the

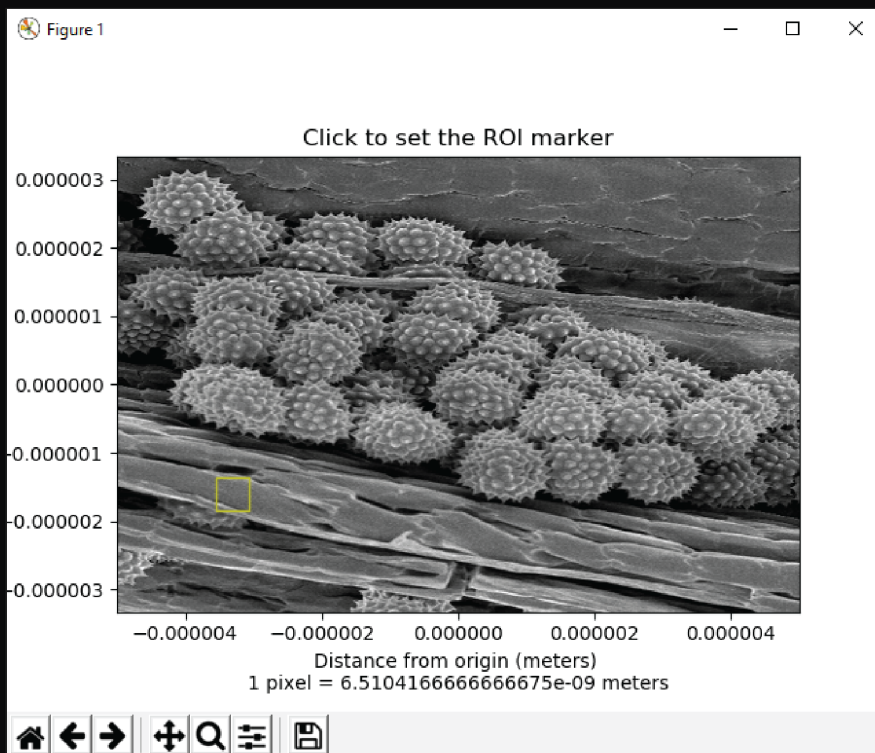
325 parameters selected are the correct ones. This screenshot demonstration has been produced using

326 the Autoscript simulation mode.

327

Anaconda Prompt - autolamella config.yml

```
FIRST MOVE TO THE DESIRED POSITION
Do you want to select a new location for milling? [y]/n
y
Please select where to put a fiducial marker.
-3.3064516129e-06 -1.60483870968e-06
```



328

329 **Supplementary figure 4: Screenshot of the interactive selection of the tracking area position.** The

330 user finds a suitable location for a lamella interactively using the ThermoFisher Xt UI™. Once the new

331 location has been identified, the user replies yes to the question in the command prompt, “Do you

332 want to select a new location for milling?”. The current ion beam image is then displayed in a pop-up

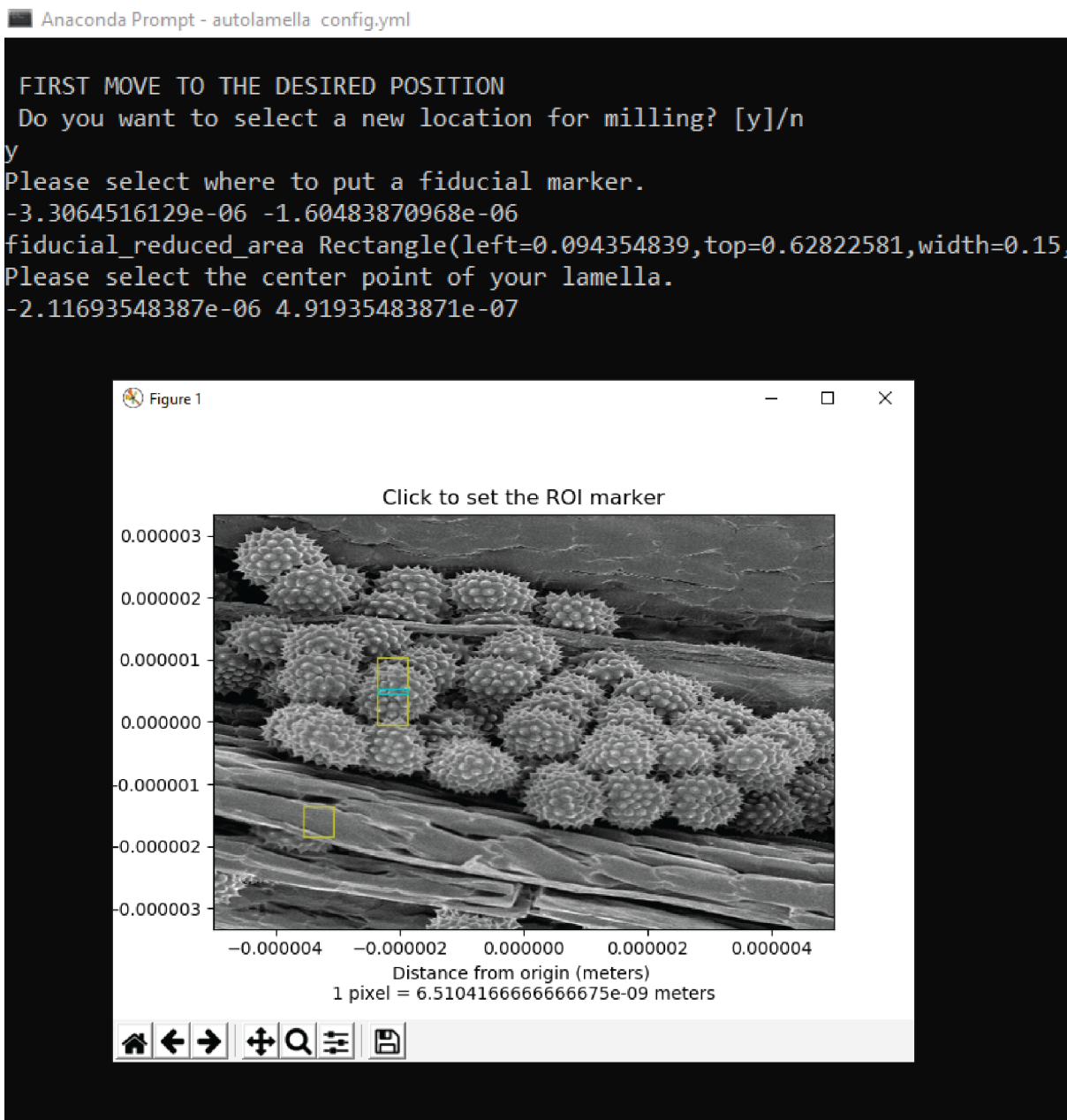
333 window, and the user clicks to select the position for the fiducial marker. The fiducial marker position

334 is indicated with a yellow box, and this position can be adjusted before closing the pop-up window.

335 Closing the window leads to continuing the process. Screenshots have been produced using Autoscript

336 simulation mode.

337



338

339 **Supplementary figure 5: Screenshot of the interactive selection of the lamella position.** The
340 command-line prompt asks the user to “Please select the centre point of your lamella” and a new pop
341 up window displays the current ion beam image with the fiducial marker position shown for reference.
342 The user clicks on the image to choose the position for the lamella. The cyan rectangle indicates the
343 position of the final lamella, and the surrounding yellow box indicates the total size of the trenches as
344 defined in the configuration file. The user can click on in multiple locations to optimise the position of
345 the lamella, and the last coordinates chosen before closing the window will be used for milling.
346 Screenshots have been produced using Autoscript simulation mode.

347


```
■ Anaconda Prompt

FIRST MOVE TO THE DESIRED POSITION
Do you want to select a new location for milling? [y]/n
y
Please select where to put a fiducial marker.
-3.3064516129e-06 -1.60483870968e-06
fiducial_reduced_area Rectangle(left=0.094354839,top=0.62822581,width=0.15,height=0.225)
Please select the center point of your lamella.
-2.11693548387e-06 4.91935483871e-07
Do you want to mill a fiducial marker here? [y]/n
y
Milling fiducial marker...
Do you want to re-mill the fiducial marker? y/[n]
n
If you want a custom milling depth for the LAMELLA enter it here in meters, else- slam on ENTER:

FIRST MOVE TO THE DESIRED POSITION
Do you want to select a new location for milling? [y]/n
y
Please select where to put a fiducial marker.
3.70967741935e-06 8.75e-07
fiducial_reduced_area Rectangle(left=0.79596774,top=0.25625,width=0.15,height=0.225)
Please select the center point of your lamella.
1.49193548387e-06 -4.5564516129e-07
Do you want to mill a fiducial marker here? [y]/n
y
Milling fiducial marker...
Do you want to re-mill the fiducial marker? y/[n]
n
If you want a custom milling depth for the LAMELLA enter it here in meters, else- slam on ENTER:

FIRST MOVE TO THE DESIRED POSITION
Do you want to select a new location for milling? [y]/n
n
Do you want to mill all samples? yes/no
yes
2019-09-17 16:05:49,659 INFO Protocol stage 1 of 3
2019-09-17 16:05:49,659 INFO Lamella number 1 of 2
2019-09-17 16:05:53,985 INFO Lamella number 2 of 2
2019-09-17 16:05:58,831 INFO Protocol stage 2 of 3
2019-09-17 16:05:58,832 INFO Lamella number 1 of 2
2019-09-17 16:06:03,225 INFO Lamella number 2 of 2
2019-09-17 16:06:09,637 INFO Protocol stage 3 of 3
2019-09-17 16:06:09,638 INFO Lamella number 1 of 2
2019-09-17 16:06:15,716 INFO Lamella number 2 of 2
Finished!
```

348

349 **Supplementary figure 6: Example output from the command prompt for an automated batch job**

350 **when preparing two lamellae.** After the fiducial mark has been prepared the program images it and

351 provides the option to mill further in case the depth selected does not provide suitable contrast on

352 one particular area. Further the lamella depth can be tuned for each position in case the sample has

353 significant variations. the value should be in meters (e.g. 1e-6 for 1 μ m), if the depth specified in the

354 configuration file is acceptable the user should not enter any value. If no more locations are selected

355 (negative answer at the question "Do you want to select a new location for milling?)" the interactive

356 section ends at the question "Do you want to mill all samples?". This screenshot demonstration has

357 been produced using the Autoscript simulation mode.

358 **Supplementary video 1: slice by slice video of the tomogram shown in figure 4.**

359

360

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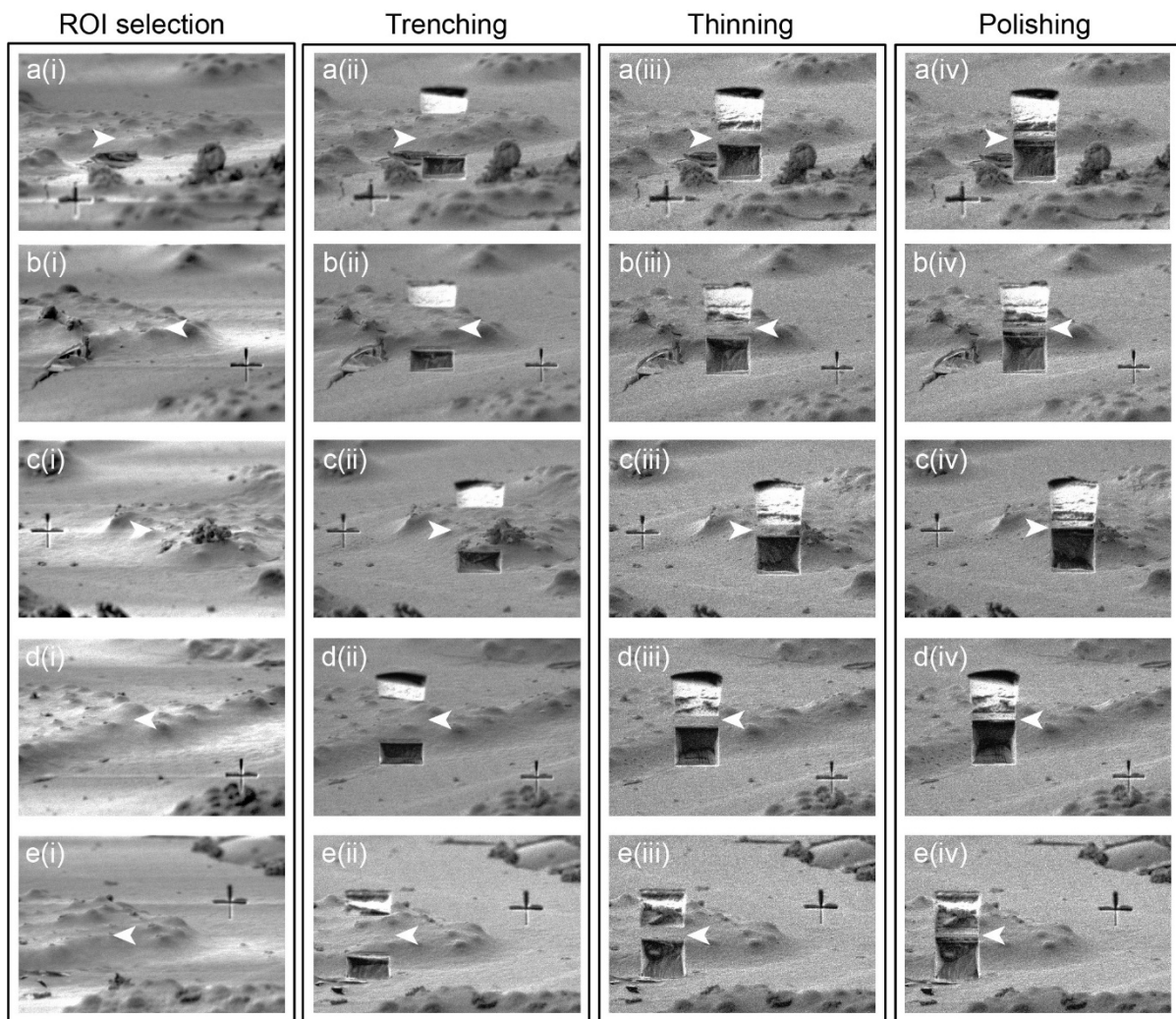
450

Supplementary Information

Automated cryo-lamella preparation for high-throughput *in-situ* structural biology

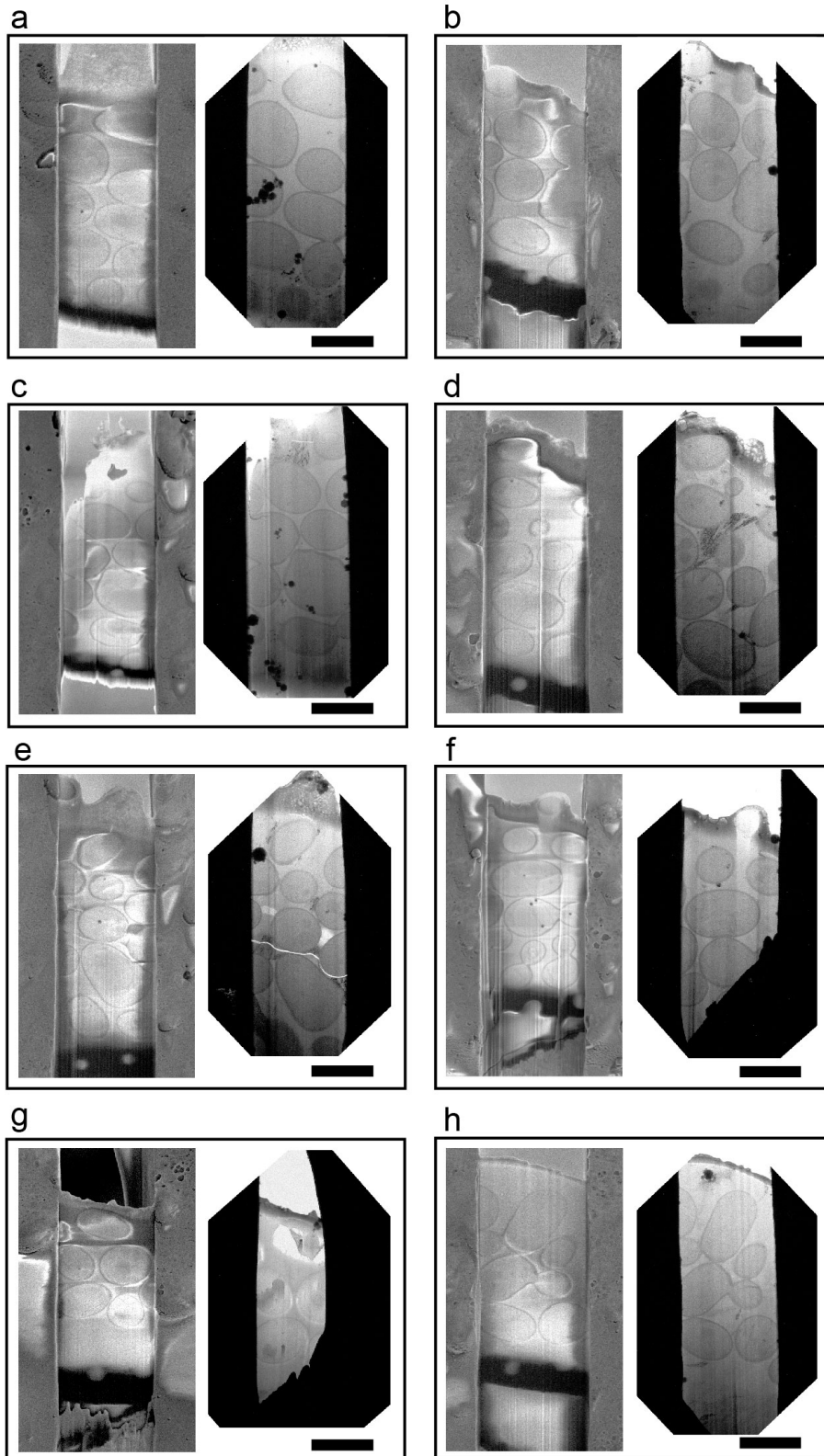
Authors:

Genevieve Buckley, Gediminas Gervinskas, Cyntia Taveneau, Hari Venugopal, James C. Whisstock, Alex de Marco



Supplementary figure 1: Ion beam images acquired throughout a single batch job consisting of 5 lamellae. The total duration of the batch job was 47 minutes, plus 12 minutes required for the initial (manual) selection of sample locations by the user. Here, each lamella location is displayed in a single row (marked a, b, c, d, e) and white arrowheads indicate the position of the finished lamella. The cross-shaped fiducial marker acts as scale-bar, with dimensions of $6 \times 6 \mu\text{m}$. Columns from left to right show: (i) the fiducial marker is created; (ii) trenches opened with high ion beam current; (iii) further thinning

with an intermediate ion beam current and (iv) the completed lamella after final polishing with low ion beam current. Ion beam milling currents for these stages were 2.4 nA, 75 pA, and 26 pA, respectively. The milling procedure runs per column, therefore, each FIB milling stage is completed for every lamella location before proceeding to the next milling stage.



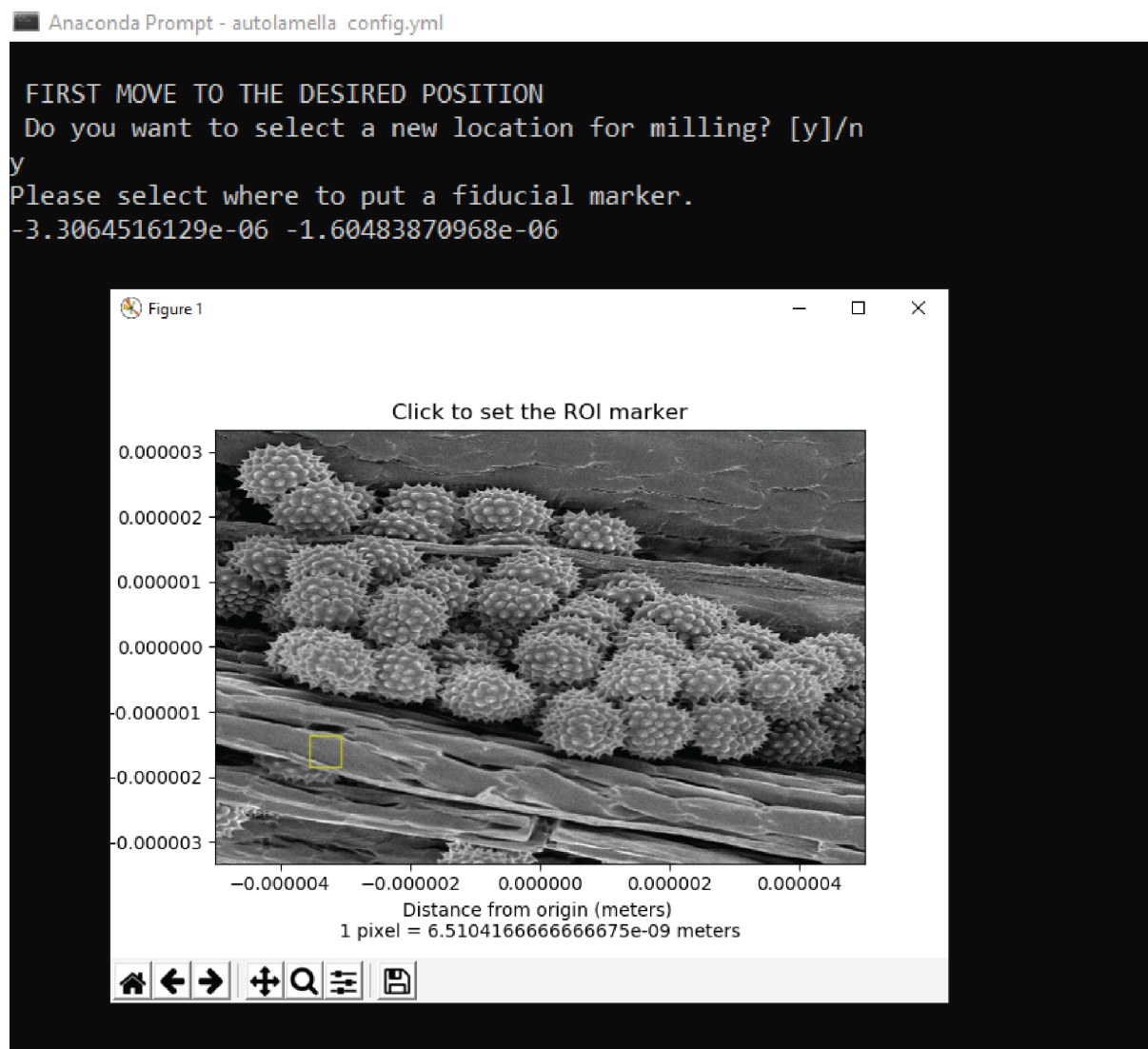
Supplementary figure 2: Gallery of 8 lamellae prepared using the procedure described in this article, illustrating quality and pitfalls. Each panel shows on the left a low voltage cryo-SEM image of the lamella surface at the end of the preparation procedure. On the right within each panel there is a low mag cryo-TEM micrograph for each lamella. The images show that the quality is comparable to current preparation approaches. In this gallery, we show potential pitfalls of batch preparation which can be easily resolved if the user is aware and careful. The level of curtaining changes in response to lamella length and surface contamination, if the sample thickness is variable the user should select more generous depth for the polishing step to ensure the surface finish is optimal. (a, b) show proper depth selection, while (c–h) show different behaviours when under-polishing). Panels (f) and (g) show that the size of the trenches was not appropriate for the sample thickness, as visible from the cryo-TEM images part of those lamellae is not electron transparent, indicating that there is bulk material underneath the lamella (also visible from the cryo-SEM). Scale-bars are 5 μm .

Select Anaconda Prompt - autolamella config.yml

```
(base) C:\Users\genevieb>conda activate fibsem
(fibsem) C:\Users\genevieb>autolamella config.yml
Create a new EMPTY directory to store your output images.
Client connecting to [localhost:7520]...
Client connected to [localhost:7520]
2019-09-17 15:52:57,754 INFO demo_mode: 1.0
fiducial:
  fiducial_image_size_x: 1.5e-06
  fiducial_image_size_y: 1.5e-06
  fiducial_length: 5.0e-07
  fiducial_milling_current: 3.0e-10
  fiducial_milling_depth: 1.0e-07
  fiducial_width: 1.0e-07
  min_distance_from_lamella: 1.0e-07
  reduced_area_resolution: 3072x2048
imaging:
  autocontrast: 0.0
  dwell_time: 1.0e-07
  full_field_ib_images: 1.0
  horizontal_field_width: 5.0e-05
  resolution: 1536x1024
lamella:
  lamella_height: 1.0e-07
  lamella_width: 5.0e-07
  milling_current: 3.0e-10
  milling_depth: 1.0e-06
  overtilt_degrees: 0.0
  protocol_stages:
  - milling_current: 3.0e-10
    milling_depth: 5.0e-07
    percentage_from_lamella_surface: 0.5
    percentage_roi_height: 0.5
  - milling_current: 3.0e-10
    percentage_from_lamella_surface: 0.2
    percentage_roi_height: 0.3
  - milling_current: 3.0e-10
    overtilt_degrees: 2.0
    percentage_from_lamella_surface: 0.0
    percentage_roi_height: 0.2
  total_cut_height: 5.0e-07
save_directory: C:/Users/genevieb/Documents/test/output_directory
system:
  application_file_cleaning_cross_section: Si
  application_file_rectangle: Si
  ip_address: localhost
```

Supplementary figure 3: Screenshot of the terminal as the user launches the ‘autolamella’ program.

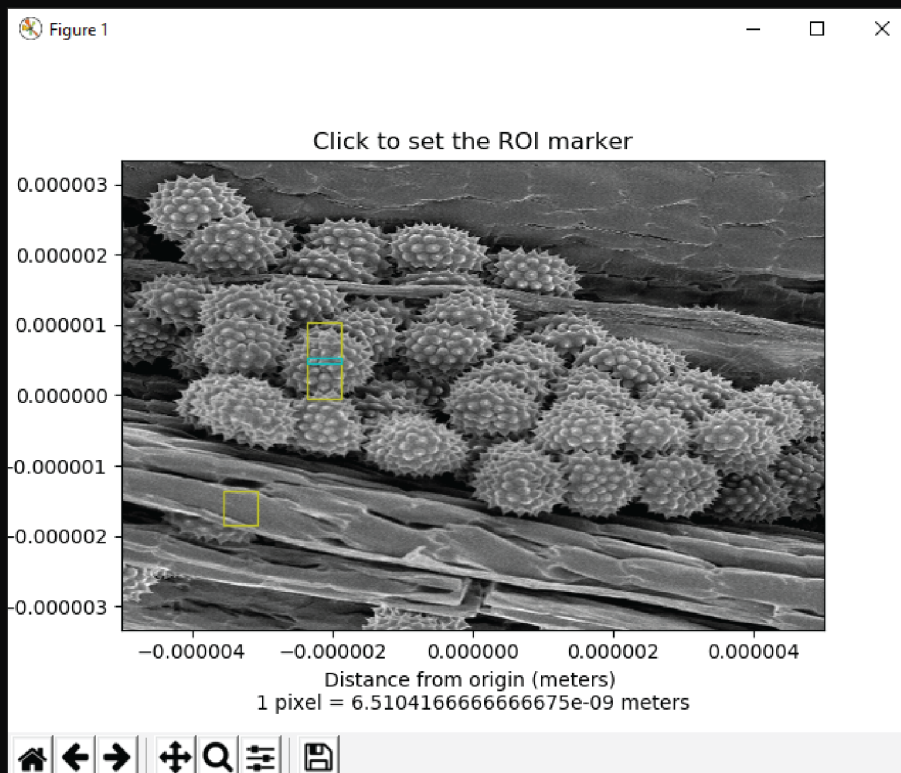
The first section of the output consists of a summary of the parameters provided in the configuration file, here named config.yml. The idea is to provide immediate feedback that the file and the parameters selected are the correct ones. This screenshot demonstration has been produced using the Autoscript simulation mode.



Supplementary figure 4: Screenshot of the interactive selection of the tracking area position. The user finds a suitable location for a lamella interactively using the ThermoFisher Xt UI™. Once the new location has been identified, the user replies yes to the question in the command prompt, “Do you want to select a new location for milling?”. The current ion beam image is then displayed in a pop-up window, and the user clicks to select the position for the fiducial marker. The fiducial marker position is indicated with a yellow box, and this position can be adjusted before closing the pop-up window. Closing the window leads to continuing the process. Screenshots have been produced using Autoscript simulation mode.

Anaconda Prompt - autolamella config.yml

```
FIRST MOVE TO THE DESIRED POSITION
Do you want to select a new location for milling? [y]/n
y
Please select where to put a fiducial marker.
-3.3064516129e-06 -1.60483870968e-06
fiducial_reduced_area Rectangle(left=0.094354839,top=0.62822581,width=0.15)
Please select the center point of your lamella.
-2.11693548387e-06 4.91935483871e-07
```



Supplementary figure 5: Screenshot of the interactive selection of the lamella position. The command-line prompt asks the user to “Please select the centre point of your lamella” and a new pop up window displays the current ion beam image with the fiducial marker position shown for reference. The user clicks on the image to choose the position for the lamella. The cyan rectangle indicates the position of the final lamella, and the surrounding yellow box indicates the total size of the trenches as defined in the configuration file. The user can click on in multiple locations to optimise the position of the lamella, and the last coordinates chosen before closing the window will be used for milling. Screenshots have been produced using Autoscript simulation mode.


```
■ Anaconda Prompt

FIRST MOVE TO THE DESIRED POSITION
Do you want to select a new location for milling? [y]/n
y
Please select where to put a fiducial marker.
-3.3064516129e-06 -1.60483870968e-06
fiducial_reduced_area Rectangle(left=0.094354839,top=0.62822581,width=0.15,height=0.225)
Please select the center point of your lamella.
-2.11693548387e-06 4.91935483871e-07
Do you want to mill a fiducial marker here? [y]/n
y
Milling fiducial marker...
Do you want to re-mill the fiducial marker? y/[n]
n
If you want a custom milling depth for the LAMELLA enter it here in meters, else- slam on ENTER:

FIRST MOVE TO THE DESIRED POSITION
Do you want to select a new location for milling? [y]/n
y
Please select where to put a fiducial marker.
3.70967741935e-06 8.75e-07
fiducial_reduced_area Rectangle(left=0.79596774,top=0.25625,width=0.15,height=0.225)
Please select the center point of your lamella.
1.49193548387e-06 -4.5564516129e-07
Do you want to mill a fiducial marker here? [y]/n
y
Milling fiducial marker...
Do you want to re-mill the fiducial marker? y/[n]
n
If you want a custom milling depth for the LAMELLA enter it here in meters, else- slam on ENTER:

FIRST MOVE TO THE DESIRED POSITION
Do you want to select a new location for milling? [y]/n
n
Do you want to mill all samples? yes/no
yes
2019-09-17 16:05:49,659 INFO Protocol stage 1 of 3
2019-09-17 16:05:49,659 INFO Lamella number 1 of 2
2019-09-17 16:05:53,985 INFO Lamella number 2 of 2
2019-09-17 16:05:58,831 INFO Protocol stage 2 of 3
2019-09-17 16:05:58,832 INFO Lamella number 1 of 2
2019-09-17 16:06:03,225 INFO Lamella number 2 of 2
2019-09-17 16:06:09,637 INFO Protocol stage 3 of 3
2019-09-17 16:06:09,638 INFO Lamella number 1 of 2
2019-09-17 16:06:15,716 INFO Lamella number 2 of 2
Finished!
```

Supplementary figure 6: Example output from the command prompt for an automated batch job when preparing two lamellae. After the fiducial mark has been prepared the program images it and provides the option to mill further in case the depth selected does not provide suitable contrast on one particular area. Further the lamella depth can be tuned for each position in case the sample has significant variations. the value should be in meters (e.g. 1e-6 for 1 μm), if the depth specified in the configuration file is acceptable the user should not enter any value. If no more locations are selected (negative answer at the question “Do you want to select a new location for milling?”) the interactive section ends at the question “Do you want to mill all samples?”. This screenshot demonstration has been produced using the Autoscript simulation mode.

Supplementary video 1: slice by slice video of the tomogram shown in figure 4.