Cryo-focused ion beam lamella preparation protocol for *in situ* Structural Biology

Jana Moravcová[1], Radka Dopitová[1], Matyáš Pinkas[1], Jiří Nováček[1]

[1] CEITEC, Masaryk University, Kamenice 5, 62500 Brno, Czech Republic

Abstract
The advances in electron cryo-microscopy have enabled high-resolution structural studies of vitrified macromolecular complexes *in situ* by cryo-electron tomography (cryo-ET). Since utilization of cryo-ET is generally limited to the specimens with thickness <500 nm, a complex sample preparation protocol to study larger samples such as single eukaryotic cells by cryo-ET was developed and optimized over the last decade. The workflow is based on the preparation of a thin cellular lamella by cryo-focused ion beam milling (cryo-FIBM) from the vitrified cells. The sample preparation protocol is a multi-step process which includes utilization of several high-end instruments and comprises sample manipulation prone to sample deterioration. Here, we present a workflow for preparation of three different model specimens which was optimized to provide high-quality lamellae for cryo-ET or electron diffraction tomography with high reproducibility. Preparation of lamellae from large adherent mammalian cells, small suspension eukaryotic cell line, and protein crystals of intermediate size is described which represents examples of the most frequently studied samples used for cryo-FIBM in life-sciences.

Key words: cryo-focused ion beam milling, cryo-electron microscopy, lamella, adherent cells, protein crystal, Saccharomyces cerevisiae

1. Introduction

Electron cryo-microscopy (cryo-EM) has been experiencing significant expansion in Structural and Cellular Biology research. This is mainly due to the technological developments in the field of electron detection [McMullan,Faruqi,Clare & Henderson 2014], the development of the electron microscopes capable of unsupervised automated data collection, and a significant progress in the development of the software for the data analysis [Zivanov et al. 2018]. Apart from the single particle cryo-EM which is now a well established technique in Structural Biology, the cryo-electron tomography (cryo-ET) is developing as a method for studies of pleomorphic samples or structural studies of macromolecular complexes *in situ [Villa,Schaffer,Plitzko & Baumeister 2013]*. Cryo-ET can reach single nanometer resolution data when applied to pleomorphic objects due to low sensitivity obtained from a single tomogram, while near-atomic resolution can be obtained through sub-volume averaging of multiple objects with the same structure [Schur 2019; O'Reilly et al. 2020]. One of the major applications of cryo-ET is the structural characterization of the macromolecular complexes in the context of other proteins and nucleic acids inside the cell. Utilization of electron tomography with the microscope set to diffraction mode opens a new application area for the structure determination from single crystals [Nannenga & Gonen 2014; Nannenga & Gonen 2016]. When implemented to protein or small organic compound crystals with thickness of ~100 nm, the technique is termed micro-ED and is nowadays rapidly developing method with significant potential in Structural Biology research.

All transmission electron microscopy (TEM) methods are limited by the sample thickness. In general, this is not any problem for single particle cryo-EM where the sample preparation can eventually be optimized to vitrify the molecules of interest into thin layer. However, more complex biological samples such as the whole cells are in fact impassable for 300 keV

electron beam which prohibits acquisition of the cryo-ET data. Similarly, micro-meter sized crystals are non-transparent for 200 or 300 keV electrons which significantly limits the utilization potential of micro-ED. In such cases, the sample must be first thinned down to render it electron transparent for the TEM imaging while maintaining it in the vitreous state. Two approaches were developed over the last decade to manage TEM imaging of the thick vitrified specimen. (1) Cryo-ultramicrotomy utilizes mechanical slicing of the sample with a diamond knife to prepare 60-80 nm cross sections from cells and tissues [Al-Amoudi,Norlen & Dubochet 2004; Al-Amoudi,Studer & Dubochet 2005; Pierson et al. 2010]. Unfortunately, the technique can result in a number of artifacts, including curved sections, crevasses, and sample compression [Al-Amoudi et al. 2005; Dubochet,Zuber,Eltsov,Bouchet-Marquis,Al-Amoudi & Livolant 2007]. Furthermore, the sections coming from the cryo-ultramicrotomy poorly attach to the TEM grids [Hsieh,Schmelzer,Kishchenko,Wagenknecht & Marko 2014]. Even though the sample is maintained in the vitreous state, the presence of the artifacts from the sample preparation may significantly limit the interpretability of the cryo-ET data. (2) Cryo-focused ion beam milling (cryo-FIBM) represents nowadays a preferred alternative for preparation of thin cross-sections from vitrified cells or protein crystals [Rigort et al. 2012; Schaffer,Engel,Laugks,Mahamid,Plitzko & Baumeister 2015]. The frozen hydrated samples are thinned by focused ion beam (FIB) of Ga^+ in a multi-step process to ablate large volume of biological material down to 80 – 300 nm thin lamella. Single lamella is obtained for each cell, representing ~0.3 – 3% of its volume, however, the methods is almost void of any sample preparation artifacts providing thus suitable input for high-resolution cryo-ET. In addition, the sample is retained on the TEM grid during the whole process which significantly facilitates the sample handling. FIB is typically combined with scanning electron microscopy (SEM) in a dual beam system, that enables simultaneous milling of the lamella with FIB and the visual inspection of the process by SEM [Villa et al. 2013]. Finally, the grid with several lamellas (typically 4-8) is transferred to TEM for cryo-ET data collection.

Still, the whole cryo-FIBM workflow combines several instruments and requires transfer between them. Each of the steps comprises a risk of the sample damage which eventually reduces the probability of obtaining high-quality lamella for cryo-ET downstream the sample preparation process. This is the major cause for relatively low throughput of the cryo-FIBM workflow.

Here, we provide an optimized protocol for the lamella preparation which comprises all steps from cultivation of the sample culture (or sample crystallization) down to the insertion of the sample into cryo-TEM for three different model specimens (Fig. 1): (1) the A9 adherent mammalian cells, (2) the *Sacharomyces cerevisiae* suspension culture and (3) the proteinase K crystals. The A9 cells represent a large eukaryotic cells which adhere onto the surface of the TEM grid and where single lamella is prepared from a single cell. The yeast cells represent small suspension cells which are applied to TEM grid before vitrification, and where single lamella is usually milled over multiple cells. The proteinase K crystals sample constitutes an example of medium-sized protein crystals which are too large for electron diffraction tomography without any further processing.

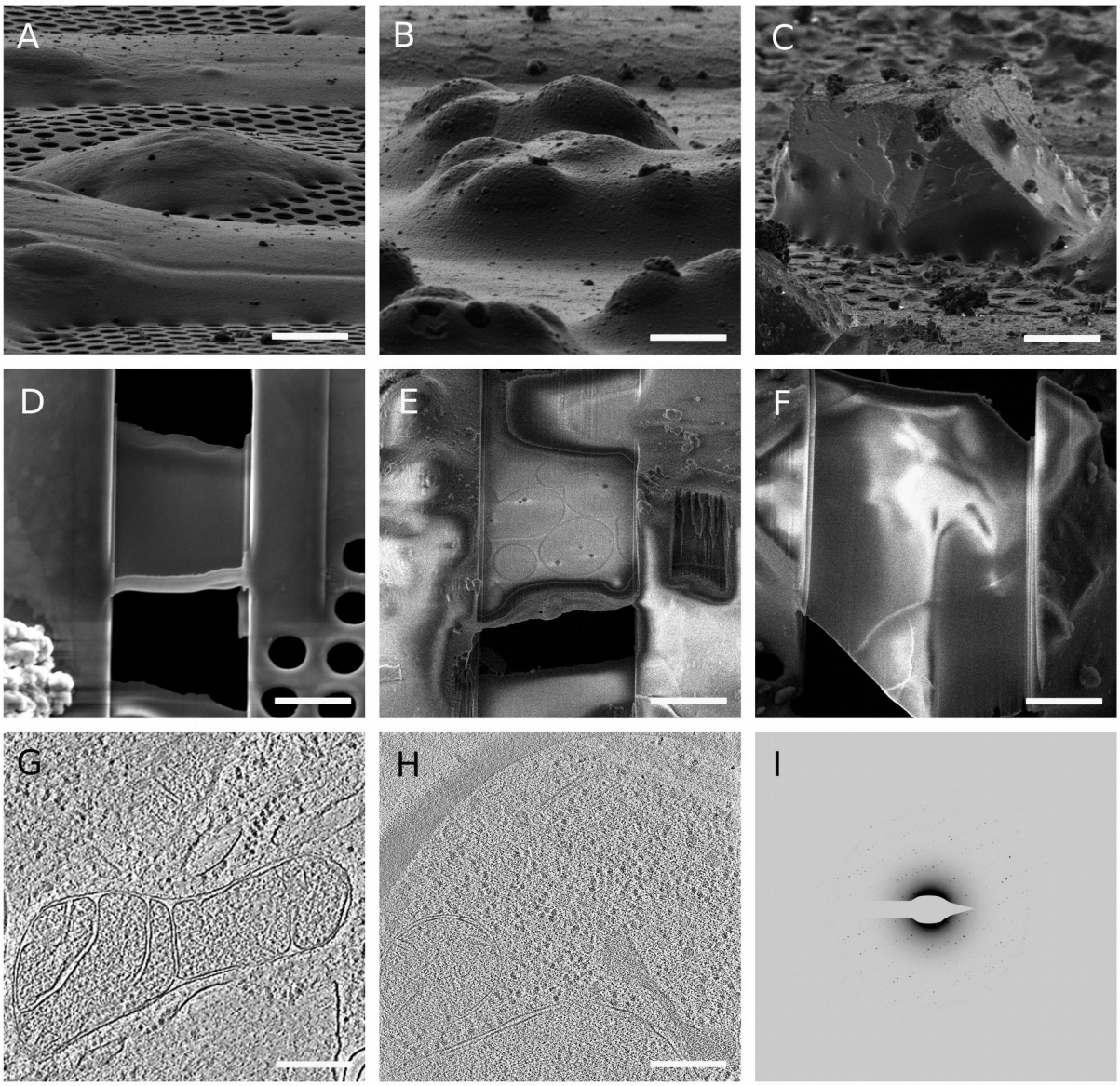

Fig. 1: FIB image of three model samples vitrified on TEM grids: adherent mammalian cell (A), *S. cerevisiae* cell cluster (B), and proteinase K crystal (C). SEM images of lamella milled from A9 cell (D), multicellular lamella from *S. cerevisiae* cell cluster (E), and proteinase K single crystal lamella (F). Section from the tomograms reconstructed from the data collected on the A9 cell (G), and *S. cerevisiae* (H) samples, respectively. Electron diffraction tomography data collected on lamella of proteinase K crystal (I).

2 Preparation of adherent cell culture samples for cryo-FIBM workflow

2.1 Materials

2.1.1 Preparation and maintaining of the mammalian adherent cell culture

Essential requirement for a cell culture work is a sterile workspace for handling, incubation, and storage of the cell culture, reagents and media. Isolated cell culture laboratory or designated workspace with cell culture hood is sufficient.
All solutions and equipment that are in contact with the cells must be sterile to avoid microbial contamination of the cell culture.

1. Biological material: *Mus musculus* A9 (APRT and HPRT negative derivative of Strain L)(ATCC® CCL-1.4™)

2. Dulbecco‘s Modified Eagle‘s Medium (DMEM) - high glucose
3. Fetal bovine serum (FBS) - inactivated by heating for 30 min at 56 °C.
4. 0.025 % Trypsin, 0.53 mM EDTA dissociation solution
5. 70 % ethanol desinfection solution
6. Dulbecco‘s phosphate buffer saline (DPBS) without Calcium chloride and Magnesium chloride
7. Plastic Petri dishes for cell culture (ø10cm)
8. Cell culture flasks (25 cm^3)
9. Micropipettes and sterile filter tips
10. Sterile microtubes
11. Serological pipette controller
12. Disposable serological pipettes
13. CO_2 humidified incubator
14. Inverted optical light microscope
15. Water bath
16. Automated Cell counter with cell counting slides or Bürker counting slide chamber
17. Laminar flow box (Class II Biological Safety Cabinet)
18. Disposable gloves

2.1.2 Preparation and vitrification of TEM grids with mammalian adherent cells

1. Plasma cleaner
2. Tweezers and precision tweezers
3. Glass microscopy slides
4. Quantifoil R2/1 or R2/4 200 Mesh Au or Cu TEM grids
5. 16 well chamber slide system (Lab Tek system or similar)
6. Vitrobot Mark IV (Thermo Scientific, Note XX)
7. Filter papers
8. Liquid nitrogen (LN2)
9. Ethane gas
10. Cryo-grid boxes
11. Grid box openers
12. LN2 Dewar bottles
13. Protective equipment for work with LN2 and ethane (glass, face shield, cold-resistant gloves)

2.2 Methods

2.2.1 Preparation and maintaining of the mammalian adherent cell culture

Following protocol is optimized for cultivation and maintaining of the mammalian adherent A9 cell line. In case of handling different cell lines, we recommend to follow the supplier instructions.

Keep standards of sterile work practice, wear gloves, use 70 % ethanol for desinfection and work in the laminar flow box.

1. Pre-warm cultivation medium, FBS, trypsin-EDTA solution to 37 °C in water bath.
2. Switch on the laminar flow box and desinfect the working surface with 70 % ethanol.
3. Spray all media, reagent bottles and equipment with 70 % ethanol before placing in the flow box.
4. Complete the cultivation medium if needed (by supplementing DMEM with FBS - 10% w/w) (Note 1).

5. Take out A9 stock aliquot from the LN2 storage Dewar and quickly thaw the cells in 37 °C water bath.
6. Spray the vial with 70 % ethanol before placing in the flow box.
7. Transfer the cell suspension into the cell culture flask, add 10 ml of the cultivation medium, and mix slowly.
8. Incubate the freshly seeded cell culture in a 37 °C, 5 % CO_2 humidified incubator.
9. Check the state of the cell culture daily in inverted optical light microscope. Monitor health, growth rate, and growth confluency (Note 2).
10. When the cells adhere to the surface of the culture flask, pipette out the cultivation medium and wash once with room temperature DPBS, add 10 ml of pre-warmed cultivation medium and return the flask back to the incubator (Note 3).
11. Once the confluency of cells reaches ~ 80 % (Note 4 and Fig. 2A), transfer the flask with cell culture from the incubator into the flow box, pipette out the cultivation medium using a sterile serological pipette.
12. Gently wash the adhered cells twice with room temperature DPBS (Note 5).
13. Remove DPBS and add 1 ml of pre-warmed trypsin-EDTA solution (Note 6) to the cell monolayer. Place the cell culture flask into the incubator and leave the cells to detach from the surface into solution for 10 minutes (Note 7). The dissociation process can be observed in an inverted light optical microscope. Trypsinization is complete, when the cells are in the suspension and own a round shape. (Fig. 2B).
14. Once most of the cells detach from the surface, place the flask back to the flow box, inhibit the trypsin-EDTA solution by addition of 2 ml of pre-warmed complete cultivation medium, and gently resuspend the cells using serological pipette.
15. Pipette the cell culture into a microtube and determine the cell count per 1 ml using automated cell counter (or Bürker counting slide chamber).
16. Add 2-3 x 10^6 cells to 10 ml of the cultivation medium in a new Petri dish and return the cell culture into the incubator. Label the Petri dish with important information e.g. cell line, passage number, date, etc. From now periodically passage the cells every 2 or 3 days until 30 passages are reaching (Note 8).

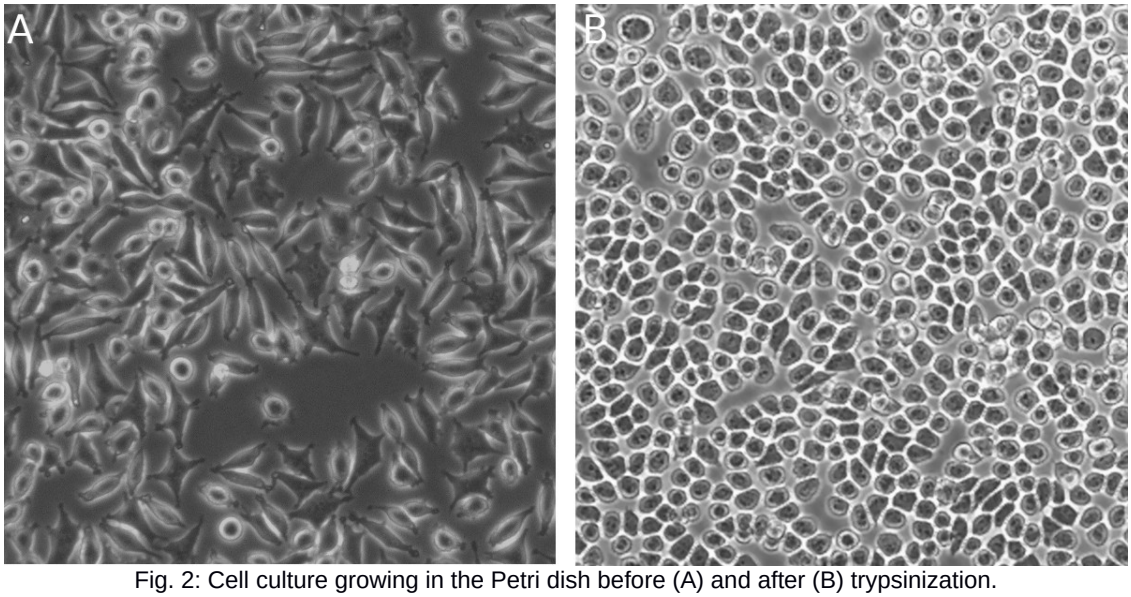

Fig. 2: Cell culture growing in the Petri dish before (A) and after (B) trypsinization.

2.2.2 Preparation of TEM grids with mammalian adherent cells

1. Place holey-carbon 200-mesh Au grids (Quantifoil Au, 200-mesh, R2/1 or R2/4) on a glass slide facing carbon side up (Note 9) and glow discharge them for 15 s (6-9 Pa

pressure, 7 mA current). Store the slide with the grids in a Petri dish until applying the cell culture (the grids should be used within one hour after plasma cleaning).

2. Transfer the glow discharged grids into the flow box for 15 min sterilization by UV.
3. Adjust a chamber slide (Fig. 3A) by removing the middle part of the chamber slide. Keep only bottom and top part of the chamber slide (Fig. 3B).
4. Place the grids into individual wells facing carbon side up with the tweezers (Note 9).
5. Add 80 ul of the complete cultivation medium per each grid, cover the chamber slide, place it in the Petri dish, and incubate the grid with the medium for approximately 1 hour (Note 10).
6. Prepare the cells according to the points 1-14 of section 2.2.1 of this protocol.
7. Dilute the cell suspension to the concentration of 1-1.5 x 10^5 cells/ml (Note 11) using pre-warmed cultivation medium. Remove the cultivation medium from the wells with the pipette. Apply 80 ul of diluted cell suspension per one grid (Note 12,13). Cover the chamber slide and place it in a Petri dish. Let the cells adhere to the surface of the grid overnight (~12-20 hours) in 37 ºC, 5 % CO_2 humidified incubator.
8. Next day, check the status of the cells in the light microscope. If the cells adhere to the grid foil (Note 14, Fig. 4A), continue with the plunge freezing.

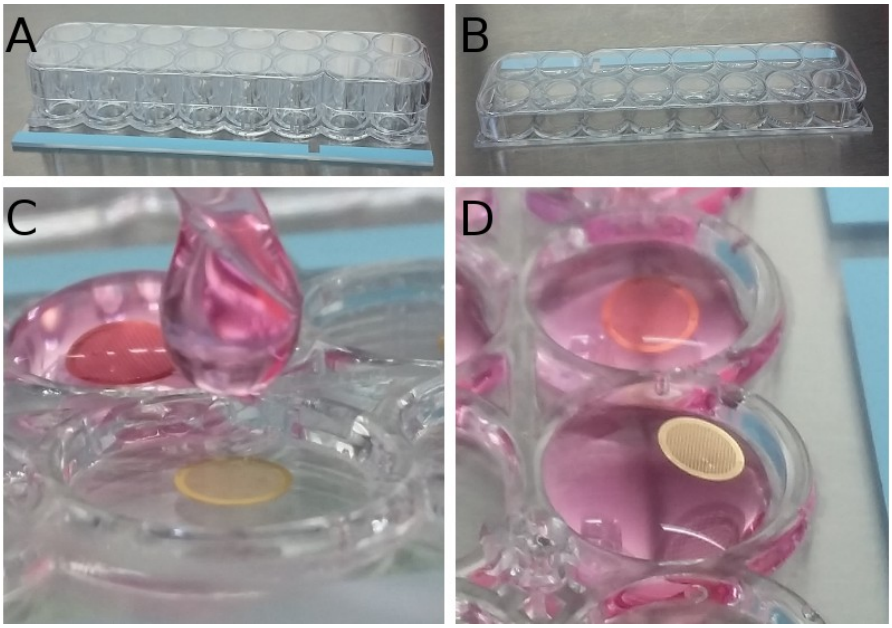

Fig. 3: Chamber slide system for adhesion of cells onto TEM grid (A). Bottom and top part of the chamber slide system used for cultivation of cells on TEM grids (B). Correct way for pipetting the cell culture onto the TEM grid (C). Grid positioned upside down on the drop of cell culture after incorrect application of the cells onto the grid (D).

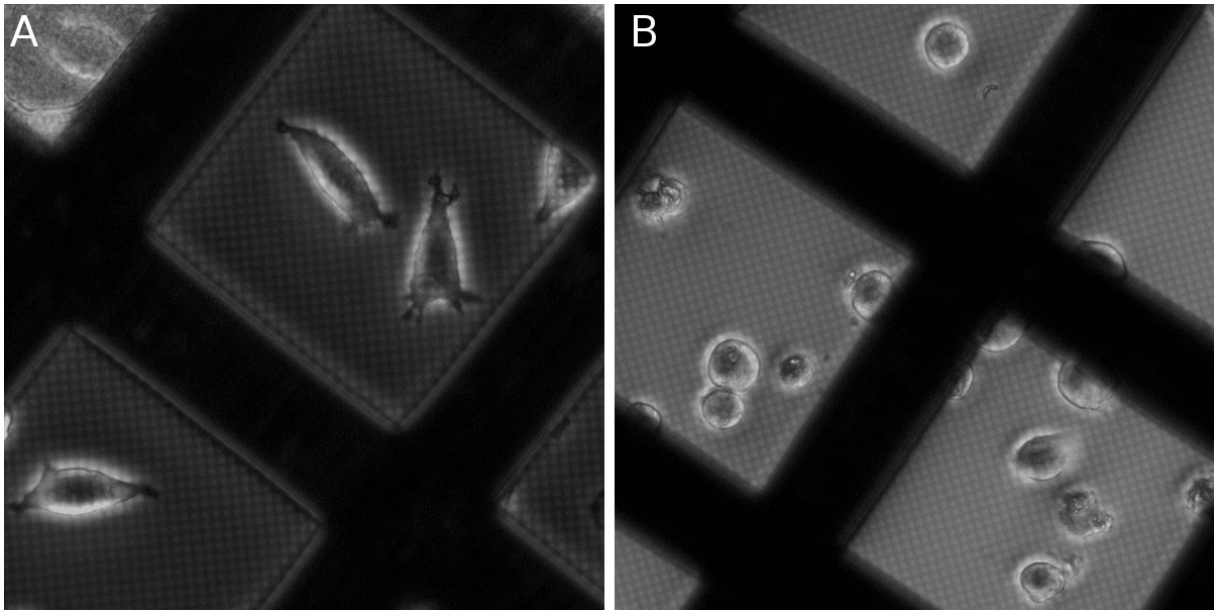

Fig. 4: A9 cells which have correctly adhered onto the TEM grid (A), dead cells on the grid (B).

2.2.3 Vitrification of the mammalian adherent cells

Sterile conditions are no longer required.
It is highly recommended to wear protective face mask or shield during vitrification procedure to prevent contamination from breathing to the specimen. Use dry tools to avoid accumulation of ice contamination.

1. Set the Vitrobot to following parameters: humidity: 100 %, temperature: 4 °C, blot force: -5, blot time: 5 s, 2 blotting cycles (Note 15), mount filter papers to both Vitrobot blotting pads.
2. Prepare liquid ethane for vitrification.
3. Wash the grid with cells twice using DPBS.
4. Mount the grid into the Vitrobot tweezers, blot the grid, and plunge immediately into liquid ethane (Note 14).
5. Store the grid in the sealed cryo-grid box under LN2 or directly clip the grid into the AutoGrid cartridge (see Chapter 5.) for loading into the FIB-SEM microscope. (Note 15).

3 Preparation of suspension cell culture samples for cryo-FIBM workflow

3.1 Materials

3.1.1 Preparation and maintaining of *Saccharomyces cerevisiae* cell culture

1. *Saccharomyces cerevisiae* cell culture (strain BY4741) (ATCC ® 201388™)
2. Sterile inoculation loops
3. Spreading sticks
4. Shaking incubator
5. UV/VIS Spectrophotometer
6. Sterile plastic Petri dishes
7. Micropipettes and sterile filter tips
8. Analytical balances

9. Disposable gloves
10. 100 – 500 ml glass bottles
11. 100 ml Erlenmayer flasks
12. Laminar flow box

Liquid growth medium for Saccharomyces cerevisiae

1. Autoclave 500 ml glass bottle for preparation of growth medium.
2. Weigh 2.2 g of Yeast extract (1.1%) and 4.4 g of Peptone (2.2%) and mix in 200 ml of Milli-Q water.
3. Sterilize by autoclaving for 15 min at 121 °C.
4. Weigh 10 g of glucose powder and mix in 50 ml Milli-Q water to get 20% glucose solution. Pass the solution through 0.2 µm filter and store it at 4 °C.

Solid medium for Saccharomyces cerevisiae

1. Weigh 4 g of agar powder and mix with 200 ml of growth medium.
2. Sterilize in autoclave for 15 min at 121 °C.
3. Cool the medium to 40-50 °C and add 20 ml of 20% sterile glucose (prepared in previous step).
4. Pour ~20 ml of the complete medium to Petri dish and let it solidify at ambient temperature.
5. Wrap agar plates by parafilm to protect drying and store at 4 °C.

3.1.2 Preparation and vitrification of TEM grids with *Saccharomyces cerevisiae* cells

The material required of the preparation and vitrification of TEM grids correspond to those listed in the section 2.1.2 (*Preparation and vitrification of TEM grids with mammalian adherent cells protocol*). In addition, PTFE (teflon) or other suitable non-adsorbing material prepared in the shape of the paper (by cutting or 3D printing) is used for sample blotting.

3.2 Methods

3.2.1 Cultivation of *Saccharomyces cerevisiae* suspension cell culture

The protocol is optimized for preparation of the sample for cryo-FIBM from suspension cell line *Saccharomyces cerevisiae* strain BY4741 [ATCC 4040002] or similar strains.

1. Autoclave 50 ml Erlenmeyer (or similar) flask.
2. Work in hood or laminar flow box.
3. Pipette 10 ml of the growth medium to sterile 50 ml Erlenmayer flask.
4. Supplement the medium with 1 ml of filtered 20% glucose.
5. Pick one colony of yeast from agar plate with sterile, disposable inoculation loop (1-10 ul).
6. Place the Erlenmayer flask to the incubator and culture at 30 °C with agitation (150-200 rpm) until exponential phase is reaching (approximately 7-15 h). (Note 16)
7. Measure optical density (OD) of *S. cerevisiae* suspension culture at 600 nm using UV/VIS spectrophotometer.
8. OD_{600} of *S. cerevisiae* culture should be in the range of 1-5 in the exponential phase of cell growth.
9. For EM grids preparation dilute the cell suspension in growth medium to have final OD_{600} = 1 (Note 17).

Cultivation of Saccharomyces Cerevisiae cell specimen on agar plate from glycerol stock stored in - 80 °C freezer

1. Take out new agar plate from 4 °C storage.
2. Pull out the *S. cerevisiae* stock from -80 °C deep freezer and place it into the freezing stand to avoid complete thawing of the stock.
3. Scrape off and transfer small culture with sterile inoculation loop (1-10 ul) to 50 ul of growth medium and mix properly.
4. Transfer whole volume of mixed *S. cerevisiae* culture and disperse with sterile spreading stick over the surface of agar plate.
5. Incubate at 30 °C for approximately 48 hours until 1.5-2 mm diameter colonies are formed.

Cultivation of Saccharomyces cerevisiase cell specimen on agar plate for keeping the culture

1. Take prepared agar plate with grown *S. cerevisiae* colonies
2. Pipette 10 ml of sterile growth medium and 1 ml of filtered 20% glucose to 50 ml Erlenmayer flask.
3. Pick one colony of *S. cerevisiae* culture with sterile inoculation loop and mix with growth medium in flask (Fig. 5).
4. Incubate 50 minutes at 30 °C with agitation (150-200 rpm).
5. Dilute the suspension culture ten times with the growth medium and disperse 50 ul of suspension on agar plate with sterile spreading stick.
6. Incubate at 30 °C for approximately 48 hours until 1.5-2 mm colonies are observed.
7. Wrap the edges of petri dish with parafilm to prevent it from drying out and store in room temperature and use for maximum 4 weeks.

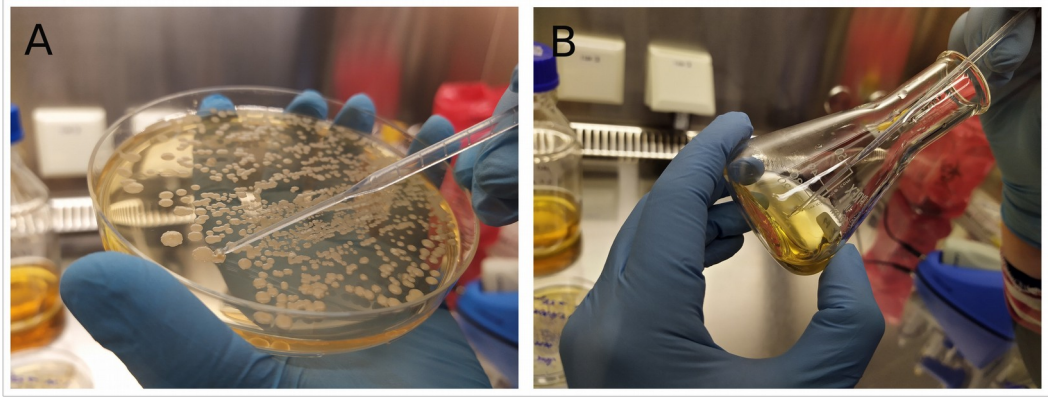

Fig. 5: Preparation of *S. cerevisiae* cell culture in liquid growing medium. Picking of the cell culture colony on solid agar plate (A) and mix with liquid growth medium (B).

3.2.2 Vitrification of Saccharomyces cerevisiae cells on EM grids

1. Prepare *S. cerevisiae* cell culture according protocol in Chapter 3.2.1 (*Cultivation of Saccharomyces cerevisiase cell culture in liquid medium*).
2. Glow discharge 200 mesh Cu or Au holey carbon grids (e.g. Quantifoil, Cu, 200 mesh, R2/1) on the glass slide facing carbon side up for 30-45 seconds (pressure: 6-9 Pa, current: 7 mA).
3. Set Vitrobot or another plunge freezing device to following parameters: temperature: 18 °C, humidity: 100%, blot time: 6 s, wait time: 5 s, blotting cycle: 1x, blot force: 5. (Note 15)
4. Prepare liquid ethane for the sample vitrification

5. Mount PTFE or different non-absorbent surface pad (0.2 mm thick) to the Vitrobot blotting pad facing the sample, use the filter paper for the other blotting pad. (Note 18)
6. Pick the glow discharged grid with the Vitrobot tweezers and mount it to the instrument. Apply 3.5 ul of *S. cerevisiae* suspension to the carbon side of the grid inside the Vitrobot climate chamber (Note 19, Fig. 6).
7. Plunge freeze the grid into the liquid ethane.
8. Store grids with vitrified cells under LN2 conditions or mount it into the AutoGrid cartridge for loading into the FIB-SEM microscope.

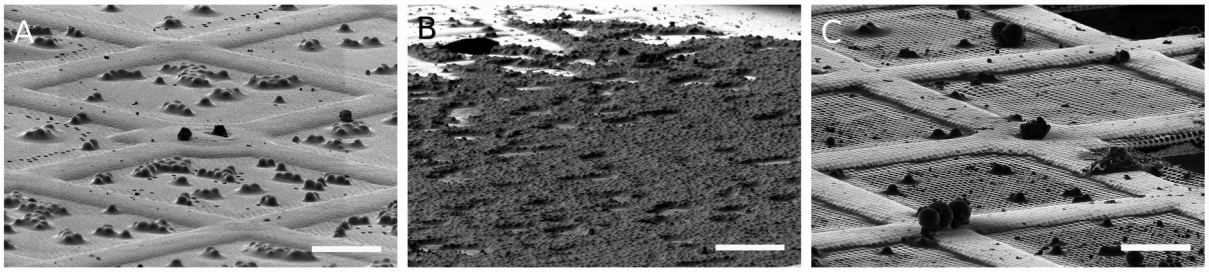

Fig. 6: Different density of *S. cerevisiae* cells plunge frozen on TEM grids. Cell clusters sufficient for multicellular lamella preparation (A). Cellular monolayer suitable for lamella preparation, although proper vitrification of the cells and residual buffer around cells may not be attained (B). Too low concentration of cells on the grid which does not allow to mill lamella (C). The scale bars correspond to 30 um in A, 60 um in B, and 30 um in panel C.

4 Preparation for protein crystal samples for cryo-FIBM workflow

4.1 Materials

4.1.1 Protein crystallization

Proteinase K crystals preparation

1. Emerald BioStructures Combi Clover Crystallization Plate™ (EBS plate) for sitting drop experiments.

Dissolving buffer and precipitating agent

1. Weigh 0.708 g of Tris-HCl powder and dissolve it in 100 ml of Milli-Q water to get 50 mM Tris-HCl solution. Adjust pH to 8 with HCl using pH meter.
2. Weigh 16.5 mg of Ammonium sulphate and dissolve in 100 ml of Milli-Q water to get 1.25 M Ammonium suplhate precipitation agent.

Proteinase K solution

1. Weigh 60 mg of proteinase K (lyophilizate powder of recombinant Proteinase K, ,purchased from Roche) using analytical balance.
2. Dissolve proteinase K powder in 1 ml of 50 mM Tris-HCl buffer pH=8.
3. Mix well by pipetting.

4.1.2 Preparation and vitrification of TEM grids with protein crystals

The materials required of the preparation and vitrification of TEM grids correspond to those listed in the section 2.1.2 (*Preparation and vitrification of TEM grids with mammalian adherent cells protocol*).

4.2 Methods

Seeding of proteinase K crystallization drops

Work in 4 °C cold room to prevent evaporation of proteinase K solution.

1. Pipette 200 ul of 1.25 M Ammonium sulfate as a precipitating agent to reservoir of crystallization plate.
2. Pipette 2 ul of 1.25 M Ammonium sulfate to small well for seeding drop in crystallization plate.
3. Add 2 ul of 60 mg/ml solution of proteinase K to small well for seeding drop and mix properly by pipetting.
4. Cover wells with transparent adhesive tape to prevent evaporation of solutions and maintain balance between mixture.
5. Incubate at ambient temperature (21-23 °C) for 2-24 h to form proteinase K crystals in the size range of 10-100 um (Notes 20, 21).

Vitrification of Proteinase K crystals on EM grids

1. Glow discharge 200 mesh Cu or Au holey carbon grids (e.g. Quantifoil, Cu, 200 mesh, R2/1) facing carbon side up for 30-45 seconds (pressure: 6-9 Pa, current: 7 mA).
2. Prepare liquid ethane.
3. Mix 4 ul of proteinase K crystals grown in small crystallization well or in the microtube with 12-16 ul of 1.25 M Ammonium sulphate (Fig. 7A,B,D,E).
4. Pick up glow discharged TEM grid with the Vitrobot tweezers and hold it in horizontal position.
5. Apply 3.5 ul of proteinase K mixture to TEM grid and blot manually with blotting filter paper from back side outside the Vitrobot chamber.
6. Mount the tweezers to Vitrobot immediately after blotting and plunge the grid in the liquid ethane.
7. Store grids with vitrified crystals in storage Dewar or clip it into the AutoGrid cartridge for loading into the FIB-SEM microscope.

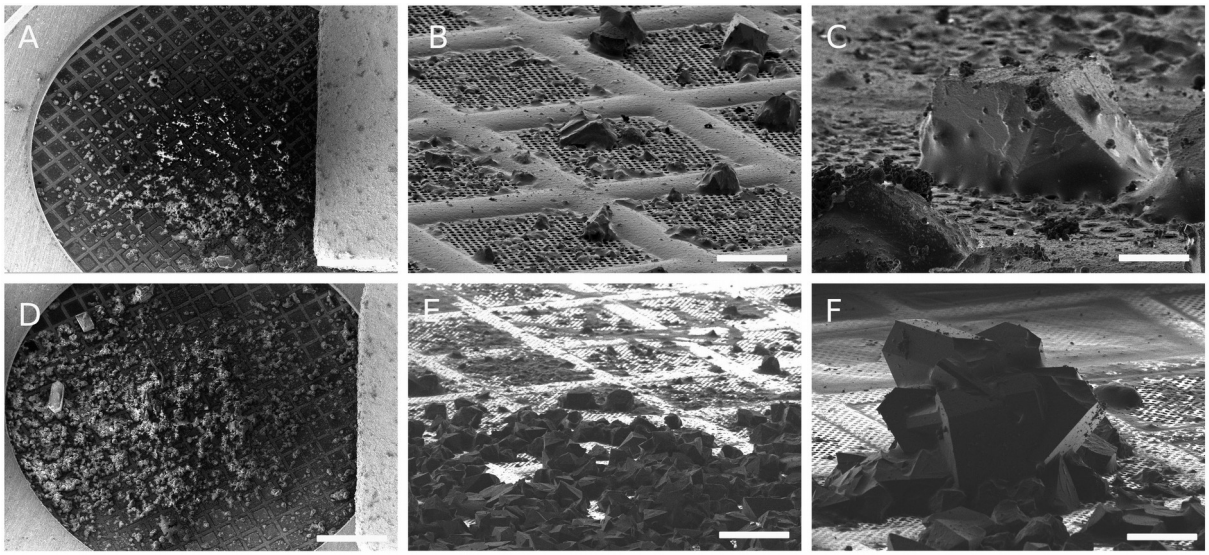

Fig. 7: SEM overview of TEM grid with plunge frozen proteinase K crystals in density suitable for FIB milling (A). FIB images of crystals dislocation on grid squares (B) and single crystal with suitable size of 25um in diameter

placed in the center of grid square (C). SEM overview image and FIB image of the overloaded TEM grid with plunge frozen proteinase K crystals (D resp. E). FIB image illustrating a cluster of proteinase K crystals with a large crystal (>100 um) not suitable for lamella preparation (F). The scale bars represent 400um in panels A and D, 30um in panels B and F, 10um in panel C, and 50um in panel E.

5 Mounting TEM grids into the AutoGrid

5.1 Materials

1. AutoGrid assembly workstation
2. AutoGrid box/boxes
3. AutoGrids
4. C-clips
5. C-clip insertion tools
6. AutoGrid tweezers
7. Grid box openers
8. Precision tweezers
9. Dewar with fresh LN2
10. Heating plate with air flow or small oven drying of tools

5.2 Methods

The workflow described here utilizes the TEM grids mounted into the AutoGrid™ (Thermo Scientific) to facilitate sample handling and transfer between SEM and TEM microscopes. Other options are available when working with the instrumentation from other microscope manufacturers.

The AutoGrid assembly workstation is filled with LN2. The LN2 level covers the gridbox with the TEM grids, but the mounting of the TEM grids into the AutoGrid cartridge is performed in LN2 vapours. It is highly recommended to wear protective face mask or shield during clipping procedure to prevent contamination from breathing to the specimen. Do not work with the tools which accumulated the ice contamination.

Loading C-clip into the clipping tool

1. Take the middle part of the C-clip with a precision tweezers and insert it vertically into the clipping tool.
2. Turn the clipping tool around and place it on a flat surface. Then, push the plunger gently down to align the C-clip properly horizontally to the edge of the clipping tool.

Assembling AutoGrids inside of clipping workstation

1. Dry all tools used for clipping to AutoGrid casette before cooling down in LN2.
2. Put the AutoGrid assembly workstation together.
3. Place the AutoGrid into the clipping metal slot with the flat side facing down. Center it properly.
4. Fill AutoGrid assembly workstation with LN2 to the level of the edge of the bottom disc. Avoid pouring LN2 on the upper disc of the workstation.
5. Place the empty AutoGrid storage box and the grid box with vitrified sample grids into appropriate slots (Note 23).
6. Unscrew the lid with pre-cooled grid box opener from the sample grid box (Note 24).
7. Cool down clipping tool with the C-clip, AutoGrid tweezer and precision tweezers (No.5)
8. Take one grid with pre-cooled precision tweezers out of the grid box and place it on the AutoGrid cartridge facing down.

9. Center the upper disc of the clipping assembly workstation over the grid using the AutoGrid tweezers.
10. Put the clipping tool with a C-clip on top of the grid and clip slowly.
11. Turn the disc back and take the AutoGrid assembly with the AutoGrid tweezer out of the slot and store it in the AutoGrid box (Note 25).
12. Place the grid on the site groove and flip it over to see if the grid is properly mounted.
13. Position the clipped AutoGrid cartridge into the AutoGrid box.
14. Continue with the clipping or close the AutoGrid box with pre-cooled lid (Note 25).
15. Store cartridges in the Dewar with LN2 or load it into the FIB-SEM microscope.

6 Sample manipulation in FIB-SEM microscope

Specimen coating with metal protective layers

Strong charging effect is observed when imaging frozen hydrated biological material SEM. In addition, imaging biological samples with FIB (even at low currents) induces fast sample damage. Therefore, additional coating of the sample is performed inside the FIB-SEM microscope, in order to protect the specimen surface and increase its conductivity.

1. Deposition of the protective layer with the Gas Injection System (GIS, Note 26).
   a. Set the sample to eucentric height
   b. Tilt the stage back to 0° (sample tilted to 45° with respect to electron beam).
   c. Move stage in *z* axis 4 mm below eucentric height (Note 27).
   d. Set the GIS needle to 26 - 30°C.
   e. Deposit ~20 nm of the GIS layer on the grid with biological specimen (Corresponds to 10 - 30 s of the GIS deposition).

2. Sputter coating of the specimen surface with conductive metal layer (Note 28).
   a. Deposit ~10 nm of the metal layer (Ir, Au, Pt, etc.) to the grid with biological specimen (Notes 29, 30).

Set microscope parameters for the lamella preparation

Set imaging and milling parameters:

1. FIB – high voltage = 30kV, current = 10pA (imaging), 10pA - 300pA (FIB-milling)
2. SEM – high voltage = 2kV – 5kV (Fig. 8), spot size = 4.5, current = 8 – 27 pA.
3. Scan rotation: 180°
4. Stage tilt - milling angle 6° - 11° (+7° stage pretilt, Note 31).

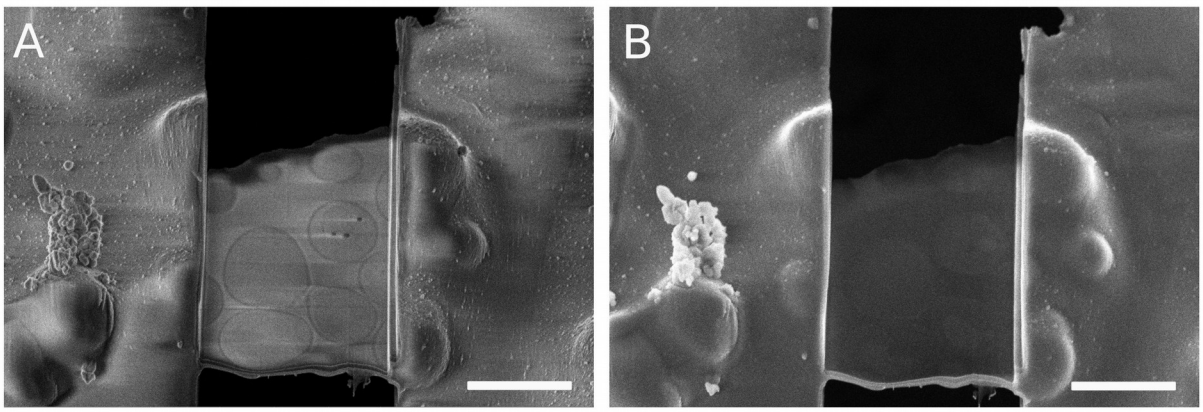

Fig. 8: Comparison of SEM imaging of *S. cerevisiae* lamella taken on ETD detector in FEG accelerating voltage 5kV (A) and 2kV (B) (scale bars = 4um).

SEM imaging of the specimen and grid quality control before FIB milling

1. Optimal cell or protein crystal concentration is reached when the specimen is individually distributed on the grid without any visible clustering (Fig. 9A-D).
2. The specimen should be positioned in the center of the grid square.(Note 32, Fig. 9E)
3. The specimen should be surrounded by visible holey carbon holes showing the ice thickness of the grid square is sufficiently thin for effective milling.
4. The cells should be free of surface ice contamination coming from air humidity or breathing.
5. The cell, cell clusters or protein crystal (Fig. 9E-F) should be at least 1.5 um high and have more than 8 um in X or Y dimension.
6. The holey carbon foil around the selectec cells should be free of cracks (Note 33).

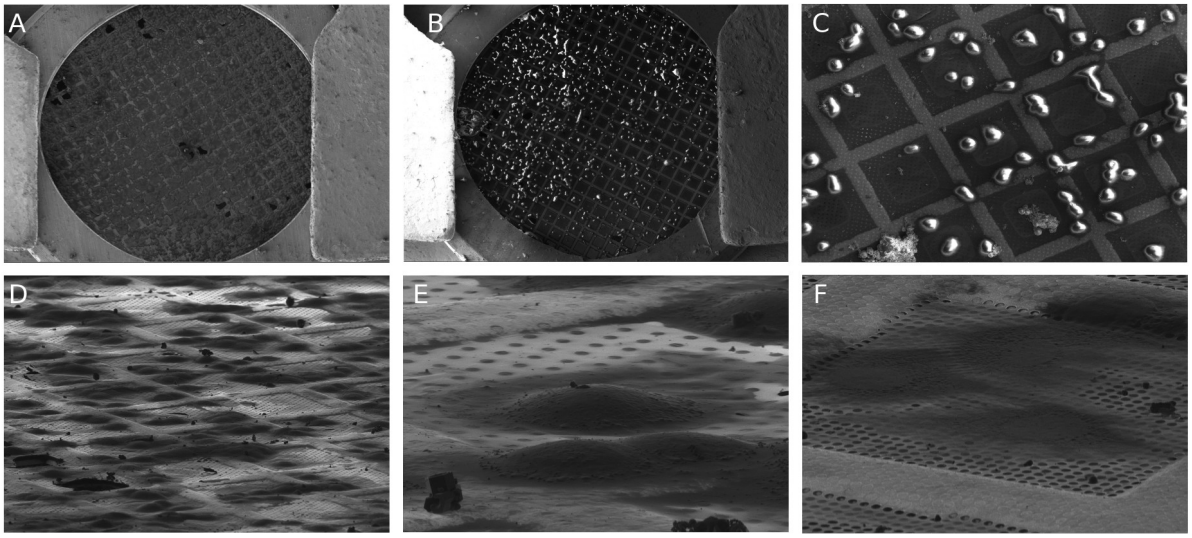

Fig. 9: Cryo-SEM images of TEM grid with vitrified A9 mammalian adherent cells. Electrom beam images of A9 cells showing overloaded grid (A) and optimal concentration of the cells on the grid (B and C in detail). Optimal concentration of the cells on the grid imaged by Ion beam. (D). Ideal positioning and spatiality of the cell (E). Grid with shrivelled cells (F).

7 Preparation of cellular or protein crystal lamella

When all requirements for the quality of the grid (see above) are fulfilled, select the object for lamella preparation.

Selection of the suitable milling object

1. Select the milling object nearby center of the grid (Note 32).
2. Select the milling object at the center of the grid square (Note 33).
3. Select the milling object at the grid square with compact holey carbon foil without cracks (Note 34).
4. Select the milling object on the grid properly blotted from both sides without additional water around milling object and on the back side of the grid (Note 34).

FIB-milling

The milling pattern is generated (Fig. 10) and centered with respect to the region of interest. Cryo-FIBM is performed sequentially with multiple milling steps performed at different FIB settings. The lamella with roughly 2 um thickness is initially milled using high current (300 pA). The lamella is subsequently gradually thinned further to the thickness of 500 nm. The fine-milling step at low current (10 pA) is used to finalize the lamella to ~200 nm thickness (Fig. 11).

1. Create upper pattern - box rectangle pattern above the region of interest with scan direction Top to Bottom.
2. Create lower pattern - box rectangle pattern below the region of interest with scan direction Bottom to Top.
3. Create middle pattern – deselected box rectangle pattern covering the region of interest providing rough estimate of the lamella thickness (this pattern is not milled during lamella preparation).
4. Mark all patterns and set the lamella width (*x* dimension). The width of the milling pattern should not exceed 2/3 of the cell width (Note 35). This corresponds to 8 – 15 um wide lamella in most of the cases
5. Set parameters for rough milling steps (Tab. 1):
   a. FIB current: 300pA; final lamella thickness: 1.5 – 2 um; width of the FIBM area: 8-12 um; stage-tilt: 13-17° (Note 31); active milling patterns: Upper and lower.
   b. FIB current: 100pA; final lamella thickness: 1 um; width of the FIBM area: 7.5 - 11.5 um; stage-tilt: 13-17° (Note 31); active milling patterns: Upper and lower.
   c. FIB current: 30pA; final lamella thickness: 0.5 um ; width of the FIBM area: 7.5 - 11.5 um; stage-tilt: 13-17° (Note 31); active milling patterns: Upper and lower.
6. Set parameters for fine milling step (Tab 1.):
   a. FIB current: 10pA; final lamella thickness: 0.2um; width of the FIBM area: 7 - 11 um; stage-tilt: 13-17° (+1°, Note 31); active milling patterns: Upper.

Milling step	Current (pA)	Overtilt	Lamella thickness (um)	Lamella width (X) shrink (um)	Upper pattern	Lower pattern
1.	300	-	1.5 - 2	0	allowed	allowed
2.	100	-	0.8 - 1	-0.5	allowed	allowed
3.	30-50	-	0.5	-0.5	allowed	allowed
4.	10	+ 1°	< 0.5	-0.5	allowed	disabled

Tab. 1: Summary of the FIB milling settings for individual steps of lamella preparation.

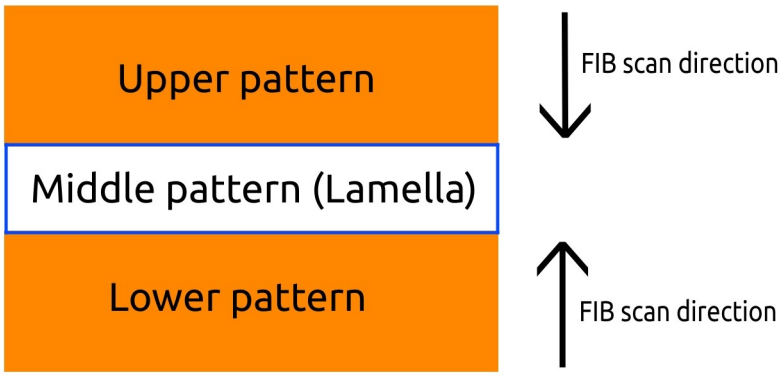

Fig. 10: Illustration of the pattern for lamella preparation. Arrows on the right side indicate recommended direction of the FIB scan direction. Dimensions for upper, middle and lower pattern in individual milling steps are listed in Table 1.

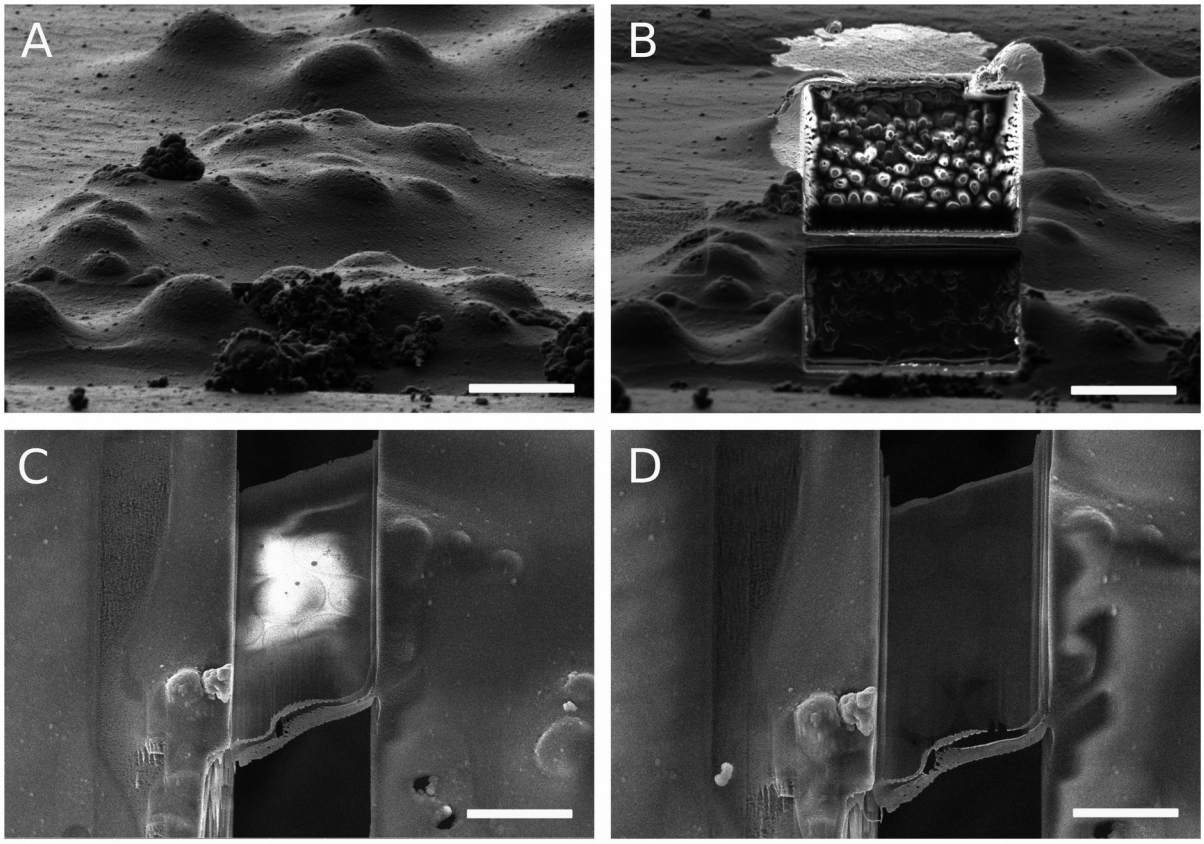

Fig. 11: FIB image of *S. cerevisiae* cell cluster plunge frozen on TEM grid and selected for lamella preparation (A). FIB image of the lamella after rough milling (B) and SEM image (C), respectively. SEM image of *S. cerevisiae* lamella polished to the final thickness of ~200nm (D). The scale bars represent 4um in panels A and B, 6um in panel C, and 5um in panel D.

Lamella transfer to cryo-TEM

1. Prepare properly dried Dewar and fill it with LN2 (Note 36).
2. Unload the samples with lamellae from the FIB-SEM microscope under cryo-conditions with care, transfer it to a grid-box, and store in LN2 storage Dewar for long term storage. Alternatively, directly load the grids into the cryo-TEM.
3. Correct orientation of the lamella with respect to the cryo-TEM stage rotation axis is important. The milling direction of prepared lamellae needs to be perpendicular to cryo-TEM stage tilt axis.

4. When using the dose-symmetric scheme (cit.) for cryo-ET data acquisition, pre-tilt the cryo-TEM stage to compensate for the lamella tilt (Note 31).
5. Assymetric angular distribution is advisable to use when collecting electron diffraction tomography data.

8 Notes

Note 1: A typical cell culture media consist of amino acids, glucose, vitamins, inorganic salts, and serum as a source of hormones, growth factors, and adhesion factors. Moreover, the media also help to keep pH and osmolality. Phenol red as a medium component serves as a pH indicator. There are also extra media supplements which can help to optimize cell growth and viability. For example, antibiotics are often used to inhibit bacterial and fungal contamination. L-glutamine is important for energy production and for protein and nucleic acid synthesis as well. 2-Mercaptoethanol as a reducing reagent is utilized to avoid toxic levels of oxygen radicals. In addition, glucose sodium pyruvate is added as a carbon source. Non-essential amino acids can replace depleted amino acids during the cell growth.

Note 2: The cells look healthy when they are mainly attached to the bottom surface of the culture flask, round and plump, or elongated in shape (A9 cells possesses elongated star-like shape), and refracting light around their membrane. Media should be pinky-orange in color. On the other hand, the dead cells detach from the surface and/or look shrivelled and dark in color or if they are in quiescence - not growing at all.

Note 3: Purpose of the medium change in this step is removal of DMSO used as a protective agent for cell freezing.

Note 4: When the cells reach ~80% confluency, they still shall be in their log phase of growth and in the best condition for passaging. It is not recommended to let the cells grow over confluency as this may negatively affect cellular expression of certain genes and viability of cells.

Note 5: The purpose of the washes with DPBS is to remove any traces of serum, calcium, and magnesium that can inhibit the Trypsin-EDTA dissociation action in the following step.

Note 6: Trypsin can be toxic to some cell lines. Consider other possibilities of cell dissociation in such case, which comprise gentle cell scraping or using a very mild dissociation reagents.

Note 7: Dissociation time can vary between cell lines. Optimize the incubation time needed for complete dissociation of the cells. However, do not expose the cells to trypsin solution for periods longer than 10 minutes, because trypsin causes damage of the cells.

Note 8: The number of passages should be recorded and shall not exceed 30. This is protection against use of the cells undergoing genetic drift and other variations.

Note 9: When using Quantifoil grids, “carbon side up” position of the grids in the chamber slide can be checked under the light microscope. The Quantifoil grids have a “1” mark at the edge. If a mirrored “1” is observed, then the grids are placed carbon side up.

Note 10: Fetal bovine serum (FBS) included in complete medium contains adhesion-promoting molecules such as fibronectin and victronectin and thus helps in adhesion of cells to the surface of the grid.

Note 11: Cell concentration used for cell adhesion on the surface of the grid have to be optimized for each cell line.

Note 12: We have found that 80 ul volume of cell suspension per well is optimal. The volumes below 50 ul are not sufficient for the cells to survive in the incubator overnight.

Note 13: Do not come too close to the grid with the pipette when applying cell suspension, as having the tip too close to the grid would cause its adhesion to the pipette tip and improper positioning of the grid at the surface of the media (Fig. 3D). Therefore, apply the cell suspension from a distance (Fig. 3C). If the grids end-up on the top of the drop facing upside down, return it back to its original position using tweezers.

Note 14: The A9 cell are adhered, when they have a star-like shape (Fig 2A.) and they are dead and not adhered to the grids when they stay round. There might be several reasons for cells not to adhere on the grids, e.g. the cell suspension was contaminated during the preparation or low volume of cell suspension was applied on the grids.

Note 15: Vitrification parameters must be experimentally determined for each instrument, because they can vary among different plunge freezing devices (even in the case of using the same type of instrument).

Note 16: Start of exponencial phase is dependent on age of culture on agar plate, use agar plates in maximal age of 6 weeks, then prepare new agar plate with *S. cerevisiae* cell culture.

Note 17: Yeast optical density OD_{600} = 1 corresponds to 10^7 cells/ml.

Note 18: Non-absorbent surface is used as an alternative material for samples adhesive to filter paper. On the other hand, filter paper on back side of the grid aspirates any excess liquid from the grid.

Note 19: To maximize quality and reproducibility of *S. cerevisiae* specimen, mix the cell suspension properly before every grid preparation. The *S. cerevisiae* cells quickly pellet to the bottom of the tube. Fig. 6 shows different cell density on prepared TEM grids.

Note 20: Proteinase K forms uniform sized crystals in the range of 5 – 10 um approximately after 2 hours of crystallization process.

Note 21: This size of crystals is suitable for micromachining of lamella in crystal clusters and collection of diffraction data in multiple places on lamella. On the other hand, crystals growing 24 hours are much larger with large variability in size (5 - 40 um). Single crystals of proteinase K suitable for FIB milling grow to the maximal size 10 - 50 um (Fig. 7C). Proteinase K rarely forms crystals >100um inappropriate for lamella preparation (Fig. 7F).

Note 22: Blot force is very instrument specific parameter. We recommend to test different blot force settings beforehand and select the optimal value.

Note 23: We recommend to carefully check the position of the TEM grid in the AutoGrid before clipping. Misalignment will cause grid deformation resulting in sample damage.

Note 24: Care should be take when opening the grid-box, as grids may eventually stick to the lid of the grid-box.

Note 25: Ice contamination increases with time. Therefore, clip maximally 8 grids at once**.**

Note 26: GIS embedded inside of the microscope chamber is used to treat the biological sample with a thin layer that protects the sample from radiation damage during FIB milling and imaging with ion beam.

Note 27: GIS contains a reservoir of organic metal compound of platinum (Pt), carbon (C), palladium (Pd) or tungsten (W) in a liquid state. Organic metal solution is injected by the micromanipulator with the needle in form of aerosol, that solidifies on the specimen surface and forms a solid layer - "crust". During milling is the layer evident as the so-called "lamella front".

Note 28: Sputter coating is a process using gaseous plasma for deposition of the metal layer to the specimen. Deposited metal creates a thin conductive layer on its surface and facilitates SEM imaging.

Note 29: The most common metals for sputter coating of biological material are gold, platinum, iridium or carbon.

Note 30: Sputter coating conditions such as current, voltage, material type, chamber pressure, and duration of sputtering determine the final thickness of the metal layer. We recommend to do a test to calibrate the sputter coater which is not equipped with the thickness sensor. Procedure: take the empty TEM grid covered with foil with defined thickness and defined size of the holes. Choose sputter conditions (e.g. sputter time 30s, pressure 8 x $l0^{-2}$ to 2 x $l0^{-2}$ mbar, sputtering voltage 0.1 - 3 kV, current 10pA) and measure thickness of the metal layer intensity loss in TEM or using electron tomography.

Note 31: Grids in the special holder "shuttle" are mounted and pre-tilted at 45° angle. When the cryo-stage in the SEM chamber is at 0° tilt angle, grid is oriented in the angle 45° to the electron beam and in -7° relative to the ion beam, respectively, in case of the Versa 3D microscope (ThermoScientific) used here. The specimen is mostly hidden behind AutoGrid edge at 0° stage tilt when imaging by FIB. It is important to choose a suitable stage tilt angle to rotate the sample visible by FIB without interfering of the AutoGrid edge. Perfectly handled and clipped grid in AutoGrid cartridge should be uniformly flat . The minimal stage tilt for milling allowed by currently used AutoGrids is 11° (4° between the FIB direction and the grid surface). However, due to imperfect geometry of the grid or in order to access milling positions closer to the AutoGrid edge, it is usually needed to choose higher tilt angles. Nevertheless, milling tilts higher than 20° are not recommended for two reasons: 1) lamella might get too short 2) the range of tilts available for tomogram collection will be much narrower.

Note 32: Milling closer to the base of the object will result in longer lamellae (sampling more of the cell volume). Objects close to the grid bars are harder to mill and will have narrower range of tilt series. Objects sitting directly on grid bars are ignored. Milling objects that satisfy all of the above criteria and are located near or at the center of grid are preferred over the objects closer to the edge of the grid.

Note 33: Any crack around the milled object would cause sample vibrations during tomographic data acquisition.

Note 34: Milled lamella have to be supported by the mass of non-milled part of cell, otherwise it is unstable and prone for bending or rupture.

Note 35: Better results of fine and smooth lamella surface without curtaining (e.g. Fig. 11D) can be achieved when the upper (not lower) part of the lamella is subjected to “fine” milling and thinning (in this case lamella is more stable); this process is carried out in parallel milling mode (both upper and lower patterns are active simultaneously), but the lower pattern is disabled.

Note 36: We have observed a reccuring phenomenon, that contamination from air humidity is readily deposited on milled lamella. Obtaining of high quality cryo-ET data on lamella covered with ice contamination is considerably more difficult or impossible.

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