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Preparation and cryo-FIB micromachining of the Saccharomyces cerevisiae for cryo-electron tomography

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Corresponding Author:	Jiri Novacek Masaryk University: Masarykova Univerzita Brno, South Moravia CZECH REPUBLIC
Corresponding Author's Institution:	Masaryk University: Masarykova Univerzita
Corresponding Author E-Mail:	jiri.novacek@ceitec.muni.cz
Order of Authors:	Jana Moravcova
	Matyas Pinkas
	Radka Holbova
	Jiri Novacek
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TITLE:

Preparation and Cryo-FIB micromachining of *Saccharomyces cerevisiae* for Cryo-Electron Tomography

AUTHORS AND AFFILIATIONS:

Jana Moravcová, Matyáš Pinkas, Radka Holbová, Jiří Nováček

Central European Institute of Technology, Masaryk University, Brno, Czech Republic

jana.moravcova@ceitec.muni.cz
matyas.pinkas@ceitec.muni.cz
radka.holbova@ceitec.muni.cz
jiri.novacek@ceitec.muni.cz

SUMMARY:

We present a protocol for lamella preparation of plunge frozen biological specimens by cryo-focused ion beam micromachining for high-resolution structural studies of macromolecules *in situ* with cryo-electron tomography. The presented protocol provides guidelines for the preparation of high-quality lamellae with high reproducibility for structural characterization of macromolecules inside *Saccharomyces cerevisiae*.

ABSTRACT:

Today, cryo-electron tomography (cryo-ET) is the only technique that can provide near-atomic resolution structural data on macromolecular complexes *in situ*. Owing to the strong interaction of an electron with the matter, high-resolution cryo-ET studies are limited to specimens with a thickness of less than 200 nm, which restricts the applicability of cryo-ET only to the peripheral regions of a cell. A complex workflow that comprises the preparation of thin cellular cross-sections by cryo-focused ion beam micromachining (cryo-FIBM) was introduced during the last decade to enable the acquisition of cryo-ET data from the interior of larger cells. We present a protocol for the preparation of cellular lamellae from a sample vitrified by plunge freezing utilizing *Saccharomyces cerevisiae* as a prototypical example of a eukaryotic cell with wide utilization in cellular and molecular biology research. We describe protocols for vitrification of *S. cerevisiae* into isolated patches of a few cells or a continuous monolayer of the cells on a TEM grid and provide a protocol for lamella preparation by cryo-FIB for these two samples.

INTRODUCTION:

Recent technological and software developments have made electron cryo-microscopy (cryo-EM) of vitrified biological specimens one of the key techniques in structural biology research in the last decade[1,2]. The preparation of a specimen for cryo-EM usually consists of the application of a purified protein or a complex of protein with nucleic acid onto the sample carrier (TEM grid), followed by removal of most of the liquid with a filter paper, and plunge freezing of the grid with the residual thin layer of a sample into liquid ethane or propane[3]. The sample is thus fixed in a thin layer (typically <80 nm) of amorphous buffer in a fully hydrated state, at near-native conditions, and without need of any chemical fixation or heavy metal contrasting. Imaging of the

structurally homogeneous specimen in the transmission electron microscope then results in data that can be used for the determination of the three-dimensional structure of the macromolecule at near-atomic resolution using a single particle analysis protocol[2]. Such an *in vitro* structure corresponds to the representation of the macromolecule under the conditions and treatment utilized during sample preparation. Although the structures determined under the *in vitro* conditions usually correspond to the fully functional state of the macromolecule, the capacity to image spatial relationships among various macromolecules inside the cell would provide an additional functional context to the structural data.

Cryo-electron tomography (cryo-ET) is used to reconstruct 3D volumes of pleomorphic objects or macromolecular complexes *in situ*[4,5]. The advantage of cryo-ET is that the three-dimensional information is obtained by imaging a single entity. However, the resolution at which individual macromolecular complexes or organelles are observed is very limited. Therefore, averaging of the macromolecules (sub-tomogram averaging, STA) with the same structure from a larger number of tomograms is necessary to reach 4-8 Å resolution models from the cryo-ET data[6,7]. It has recently been shown that cryo-ET and STA can also be applied to determine high-resolution structures of macromolecular machines such as ribosomes in the context of the cellular environment[7]. However, the utilization of transmission electron microscopy is limited by the specimen thickness. In general, this is not a problem for single-particle cryo-EM where optimization of the vitrification conditions can eventually result in the embedding of the sample in a thin layer of ice. On the other hand, most of the cells are not in fact electron transparent for the 300 keV electron beam. The inelastic mean free path in the vitrified biological specimens for the 300 keV electrons is approximately 395 nm[8], which means that cryo-ET studies are limited to the cellular periphery for most of the cells.

Different techniques were developed to thin the sample down to a sufficient thickness for cryo-ET. Cryo-ultramicrotomy utilizes mechanical slicing of the sample with a diamond knife at the liquid nitrogen temperature (-196 °C) to provide 60-80 nm thick sections suitable for cryo-ET[9-11]. Multiple sections can be prepared from a single cell and the data analysis can eventually produce 3D structural information for the larger part of the cell. However, the mechanical slicing can cause several artifacts such as curved sections, crevasses, or sample compression, which may influence the resulting structure and bias the cryo-ET data[10-12]. Cryo-focused ion beam micromachining (cryo-FIBM) represents an alternative approach where a thin cellular section is prepared by gradual ablation of the specimen using a focused beam of Ga+ ions (FIB) in a multi-step process, which can result in 80–300 nm thick cellular cross-section (lamella)[13-15]. In contrast to cryo-ultramicrotomy, only one lamella is prepared from a single cell, which represents ~0.3–3% of its volume, and micromachining of multiple cells is usually necessary to find a region of interest in the milled cross-section. In addition, the throughput of the whole workflow is nowadays still fairly low, often limited to 6–8 high-quality lamellae from an 8-hour cryo-FIBM session. On the other hand, the cryo-FIBM cross-sections are devoid of any compression artifacts and provide suitable input for high-resolution cryo-ET. Moreover, the transfer of the lamella to the sample carrier for cryo-ET is not necessary as the sample is retained on the TEM grid during the whole lamella preparation process and the same grid can be subsequently transferred to TEM. We expect that the throughput of the cryo-FIBM will be significantly improved soon, primarily from availability

of software for unsupervised lamella milling[16,17] and utilization of FIBs operating on the principle of charge-couple plasma, which will afford faster material ablation.

Saccharomyces cerevisiae (yeast) are eukaryotic cells of spherical shape and diameter of ~2-5 µm. Thanks to its size, accessibility, genetics, generation time, and simple manipulation, the yeast is extensively studied as a eukaryotic model organism in cellular and molecular biology research similar to *Escherichia coli*, which is well studied as a prokaryotic model organism in bacteriology. The yeast can be easily cultured in suspension and a high quantity of cells is generated in a short time (doubling time 1 – 2 hours). More importantly, yeast shares a complex internal cellular structure with animal and plant cells while retaining a small genome comprised of a low content of non-coding DNA. Structural characterization of the yeast proteome from the high-resolution *in situ* data can thus help to provide a mechanistic description for the extensive amount of functional data available in the literature.

Herein, we provide a comprehensive protocol for the acquisition of *in situ* cryo-ET data on the yeast sample, which covers all steps from the sample cultivation down to the cryo-FIBM lamella preparation, and the specimen transfer to TEM for cryo-ET data acquisition.

PROTOCOL:

1. Cultivation and preparation of *Saccharomyces cerevisiae* cells for vitrification

1.1. Prepare liquid growth medium for *Saccharomyces cerevisiae*.

1.1.1. Autoclave a 500 mL glass bottle for the preparation of growth medium.

1.1.2. Weigh 2.2 g of yeast extract (1.1%) and 4.4 g of Peptone (2.2%) and mix in 200 mL of water.

1.1.3. Sterilize by autoclaving for 15 min at 121 °C.

1.1.4. Weigh 10 g of glucose powder and mix in 50 mL of water to get 20% glucose solution. Pass the solution through a 0.2 µm filter and store it at 4 °C.

1.2. Prepare solid medium for *Saccharomyces cerevisiae*.

1.2.1. Weigh 4 g of agar powder and mix with 200 mL of growth medium.

1.2.2. Sterilize in an autoclave for 15 min at 121 °C.

1.2.3. Cool the medium to 40-50 °C and add 20 mL of 20% sterile glucose (prepared in the previous step). Pour ~20 mL of the complete medium into the Petri dish and let it solidify at ambient temperature.

1.2.4. Wrap the agar plates in parafilm to protect from drying and store at 4 °C.

1.3. Culture *Saccharomyces cerevisiae* in suspension

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

1.3.1. Autoclave a 50 mL Erlenmeyer (or similar) flask.

1.3.2. Work in a hood or laminar flow box. Pipette 10 mL of the growth medium to a sterile 50 mL Erlenmeyer flask.

1.3.3. Supplement the medium with 1 mL of filtered 20% glucose. Pick one colony of yeast from an agar plate with a sterile, disposable inoculation loop (1-10 µL).

1.3.4. Place the Erlenmeyer flask in the incubator and culture at 30 °C with agitation (150-200 rpm) until the exponential phase is reached (approximately 7 h).

NOTE: We have observed that the exponential phase is reached after ~15 h when colonies are picked from agar plates that have been cultured at ambient temperature for 4 weeks. See also Note below.

1.4. Culture *Saccharomyces cerevisiae* on an agar plate from glycerol stock.

1.4.1. Use a new agar plate from the 4 °C storage.

1.4.2. Take the *S. cerevisiae* stock from the -80 °C deep freezer and place it into a freezing stand to avoid complete thawing of the stock.

1.4.3. Scrape off and transfer a small culture with a sterile inoculation loop (1-10 µL) to 50 µL of growth medium. Mix properly.

1.4.4. Transfer the whole volume of mixed *S. cerevisiae* culture and disperse with a sterile spreading stick over the surface of the agar plate.

1.4.5. Incubate at 30 °C for approximately 48 hours until 1.5-2 mm diameter colonies are formed.

NOTE: It is advisable to culture the colonies freshly before the experiment. Colonies older than 1 week will require a prolonged period for cultivation in liquid media to reach the exponential growth phase.

1.5. Culture *Saccharomyces cerevisiae* on an agar plate.

1.5.1. Use prepared agar plates with grown *S. cerevisiae* colonies.

1.5.2. Pipette 10 mL of sterile growth medium and 1 mL of filtered 20% glucose to a 50 mL Erlenmeyer flask.

1.5.3. Pick one colony of *S. cerevisiae* culture with a sterile inoculation loop and mix with a growth medium in a flask.

1.5.4. Incubate 50 minutes at 30 °C with agitation (150-200 rpm).

1.5.5. Dilute the suspension culture ten times with the growth medium and disperse 50 µL of suspension on an agar plate with a sterile spreading stick.

1.5.6. Incubate at 30 °C for approximately 48 hours until 1.5-2 mm colonies are observed.

1.5.7. Wrap the edges of the Petri dish with parafilm to prevent it from drying. Store at room temperature and use for a maximum of 4 weeks.

1.6. Prepare *Saccharomyces cerevisiae* for plunge freezing in cell clusters.

1.6.1. Prepare the *S. cerevisiae* cell culture according to the protocol in the section *Cultivation of Saccharomyces cerevisiae in suspension* (Section 1.3) and incubate ~7 h at 30 °C with agitation (150-200 rpm).

1.6.2. Measure the OD of *S. cerevisiae* cell suspension culture at 600 nm using a UV/Vis spectrophotometer.

1.6.3. Concentrate the cell suspension to OD_{600} = 1.

1.7. Prepare *S. cerevisiae* for plunge freezing in a cell monolayer.

1.7.1. Prepare *S. cerevisiae* cell culture according to the protocol in the section *Cultivation of Saccharomyces cerevisiae suspension cell culture* and incubate ~7 h at 30 °C with agitation (150-200 rpm).

1.7.2. Measure the OD of *S. cerevisiae* cell suspension culture at 600 nm using an UV/Vis spectrophotometer.

1.7.3. Transfer the cells in medium to the centrifuge tube and gently spin for 2 minutes at a relative centrifugal force (900 x g).

1.7.4. Discard the medium from the cell pellet by pipetting.

1.7.5. Add fresh medium to the cell pellet. Calculate the volume of medium to get a cell suspension of OD_{600} equaling 30 to 60.

1.7.6. Add glycerol (50% stock solution) to the cell suspension to a final concentration of 5% shortly before vitrification.

NOTE: Glycerol works as a protective agent, which improves the quality of the ice in the regions between the cells. Glycerol is added to cells only shortly before vitrification to minimize its uptake into the cell.

2. Vitrification of the *Saccharomyces cerevisiae* specimen

2.1. Glow discharge TEM grids with the carbon film side facing up for 30-45 s (pressure: 6-9 Pa, current: 7 mA).

2.2. Set the vitrification robot to the following parameters: temperature: 18 °C, humidity: 100%, blot time: 6 s, wait time: 5 s, blotting cycle: 1x, and blot force: 5.

NOTE: Blot force is an instrument-specific value and optimal blot force values may differ among different machines. The optimal value for a particular plunger must be experimentally confirmed.

2.3. Prepare liquid ethane for vitrification.

2.4. Mount the non-absorbent surface pad to the blotting pad facing the sample. Use the filter paper for the other blotting pad.

2.5. Pick the glow discharged grid with the tweezers and mount the tweezers to the plunge freezing instrument.

2.6. Apply 3.5 µL of *S. cerevisiae* suspension to the carbon side of the grid inside the plunger climate chamber. Mix properly before every application on the grid.

2.7. Plunge freeze the grid into the liquid ethane.

2.8. Transfer the grid from liquid ethane to LN_2. Store grids with vitrified cells under LN_2 conditions or mount them into the TEM grid cartridge for loading into the FIB-SEM microscope.

3. Mounting TEM grids into the grid cartridge

NOTE: The workflow described here utilizes the TEM grids mounted into the AutoGrid cartridge to facilitate sample handling and transfer between SEM and TEM microscopes. The cartridge assembly consists of a C-ring, a TEM grid, and a C-clip ring. Other options are available when working with instrumentation from other microscope manufacturers. The AutoGrid assembly workstation is filled with LN_2. The LN_2 level covers the grid box with the TEM grids, but the

mounting of the TEM grids into the cartridge is performed in LN_2 vapors. It is highly recommended to wear a protective facemask or shield during the vitrification procedure to prevent contamination from breathing to the specimen. Do not work with the tools that have accumulated ice contamination.

3.1. Put the grid cartridge assembly workstation together and prepare dry tools for clipping.

3.2. Cool the assembly workstation down with LN_2. Cool the clipping tools to the LN_2 temperature.

3.3. Place a grid box with a vitrified sample into the assembly workstation.

3.4. Place a TEM grid with the specimen with cells facing down to the C-ring and secure with a C-clip.

3.5. Place the clipped cartridges into the grid box and close properly.

3.6. Store cartridges in the LN_2 Dewar or load them into the FIB-SEM microscope.

4. **Loading and manipulation of the sample to the FIB-SEM microscope**

NOTE: The instructions were written for the utilization of the dual-beam microscope Versa 3D equipped with the PP3010 cryo-FIB/SEM preparation system. Alternative solutions may require different specific parameters; however, the general concept of the workflow should be still valid.

4.1. Cool the microscope down to the liquid nitrogen temperature.

4.1.1. Pump the microscope chamber and preparation chamber (if present) to a high vacuum before the start of cooling down ($< 4 \times 10^{-4}$ Pa) to prevent contamination growth.

4.1.2. Set the nitrogen gas flow to 5 L/min for the preparation chamber, microscope stage, and microscope anticontaminator. Wait until all components reach a temperature of < -180 °C.

NOTE: The FIB-SEM microscope setup used in this study utilizes cooled nitrogen gas to cool down its stage and anticontaminator. Other systems may use a different method for stage cooling. The chamber pressure reaches ~3×10^{-5} Pa once the stage and anticontaminator are at -190 °C on the instrument used here. The growth rate of the hydrocarbon contaminating layer on the surface of the lamella with the experimental setup used in this study is ~15 nm/hour.

4.2. Load the grid cartridge with the specimen to the microscope.

4.2.1. Assemble and cool the loading station to the LN_2 temperature.

4.2.2. Place the shuttle (sample holder), the grid box with the specimen, the grid box opener, and tweezers into the cooled loading station.

4.2.3. Carefully transfer the grid cartridge with the specimen facing up into the shuttle.

4.2.4. Flip the shuttle inside of the loading station to the loading position.

4.2.5. Load into the microscope.

4.3. Optionally, coat the specimen with metal protective layers.

NOTE: A strong charging effect can be observed when imaging frozen hydrated biological material SEM. In addition, imaging biological samples with FIB (even at low currents) induces fast sample damage. Therefore, an additional coating of the sample might performed inside the FIB-SEM microscope, to protect the specimen surface.

4.3.1. Deposit the protective layer with organometallic platinum by the gas injection system (GIS).

4.3.1.1. Set the sample to the eucentric height (coincidence point for imaging with electrons and ions).

4.3.1.2. Tilt the stage back to 45° (sample tilted 90° relative to electron beam).

4.3.1.3. Move the stage in the *z*-axis 4 mm below the eucentric height.

4.3.1.4. Set the GIS needle to 26–30 °C.

4.3.1.5. Deposit ~300-1000 nm of the organometallic platinum layer to the grid with the biological specimen (corresponds to 30–120 s of the GIS deposition).

NOTE: We usually apply GIS for 30 s for samples with small cellular clusters and 45 s for samples with a monolayer of the cells.

4.3.2. Sputter coat the specimen surface with a conductive metal layer.

4.3.2.1. Deposit ~10 nm of the metal layer (Ir, Au, Pt) to the grid with a biological specimen.

4.4. Set microscope parameters for the lamella preparation

4.4.1. For FIB, use the following parameters: high voltage = 30 kV, current = 10 pA (imaging), 10 pA–3 nA (FIB-milling)

4.4.2. For SEM, use the following parameters: high voltage = 2–5 kV, spot size = 4.5, current = 8–27 pA.

4.4.3. Set the scan rotation to 180° for both beams.

4.4.4. Set the stage tilt: milling angle 6-11° (corresponds to stage tilt of 13°–18° for the sample shuttle with pre-tilt of 45° and FIB/SEM microscope with 52° angle between SEM and FIB column).

5. **Preparation of the *Saccharomyces cerevisiae lamella***

5.1. Check the grid quality and select a suitable cluster of *Saccharomyces cerevisiae*.

5.1.1. Check that the TEM grid is properly blotted from both sides without additional water around the cell cluster or at the back side of the grid.

NOTE: In order to check the backside of the grid, rotate the stage to -10° and image the grid with FIB.

5.1.2. Select the optimal cell clusters for lamella preparation according to the following recommendations.

5.1.2.1. Position the cell clusters in the grid center. The milling area should not extend outside of the square with dimensions 1100 x 1100 µm positioned in the center of the grid (550 µm in each direction from the grid center).

5.1.2.2. Position the cell clusters in the central part of the grid square with no overlap to the grid bar.

5.1.2.3. Position the cell clusters in the grid square with compact holey carbon foil without cracks.

5.1.2.4. Ensure that the cluster is not surrounded by ice contamination.

5.2. Select the optimal milling position in the *Saccharomyces cerevisiae* monolayer.

5.2.1. Ensure that the grid bars surrounding the cell monolayer on the selected grid square are visible.

5.2.2. Select the optimal position in the cellular monolayer according to the following recommendations. The selected areas for milling should satisfy the following criteria:

5.2.2.1. Have the region of interest in the grid center. The milling area should not extend outside the square of dimensions 1100 x 1100 µm positioned in the grid center (550 µm in each direction from the center of the grid).

5.2.2.2. Have the region of interest in the central part of the grid square with no overlap with the grid bar.

5.2.2.3. Do not surround the cell monolayer with ice contamination.

5.3. Prepare *S. cerevisiae* lamella with cryo-FIB.

NOTE: The milling pattern is generated and centered relative to the region of interest. Cryo-FIBM is performed sequentially with multiple milling steps performed at different FIB settings. The lamella with roughly 2 µm thickness is initially milled using high current (0.3–3 nA). The lamella is then gradually thinned to 500 nm. The fine-milling step at low currents (10-30 pA) is used to finalize the lamella to ~100–200 nm thickness.

5.3.1. Set a region of interest (ROI) to the eucentric height and save this position.

NOTE: The coincidence point needs to be determined and saved for every position separately. The eucentric height is set by tilting the stage to 0° and centering on the ROI by moving the stage in the *x* and *y* direction. The stage is then tilted to 25° and the ROI is brought back to the center of the scanned area by changing the stage *z*-axis position. Finally, the stage is tilted back to 13°–18° for milling.

5.3.2. Define a rectangular milling pattern above the ROI with a scan direction of Top to Bottom.

5.3.3. Define a rectangular milling pattern below the ROI with a scan direction of Bottom to Top.

5.3.4. Define the inactive rectangular milling pattern covering the region of interest for a rough estimation of the lamella thickness. This pattern is not milled during lamella preparation.

5.3.5. Mark all patterns and set the lamella width (*x* dimension). The width of the milling pattern should not exceed 2/3 of the cluster width. This corresponds to 8–15 µm in most cases.

5.3.6. Set parameters for rough milling steps

5.3.6.1. FIB current: 0.3–3.0 nA ; final lamella thickness: 1.5–2 µm; width of the FIBM area: 8-12 um; stage-tilt: 13-17°; duration: 8 minutes; active milling patterns: upper and lower.

5.3.6.2. FIB current: 0.3–1.0 nA; final lamella thickness: 1 µm; width of the FIBM area: 7.5-11.5 µm; stage-tilt: 13-17°; duration: 8 minutes; active milling patterns: upper and lower.

5.3.6.3. FIB current: 100–300 pA; final lamella thickness: 0.5 µm ; width of the FIBM area: 7.5-11.5 µm; stage-tilt: 13-17°; duration: 8 minutes; active milling patterns: upper and lower.

5.3.6.4. FIB current: 30–100 pA; final lamella thickness: 0.3 µm ; width of the FIBM area: 7.5-11.5 µm; stage-tilt: 13-17°; duration: 8 minutes; active milling patterns: upper and lower.

5.3.7. Set parameters for the fine milling step:

5.3.7.1. FIB current: 10–30 pA; final lamella thickness: <0.2 um; width of the FIBM area: 7-11 µm; stage-tilt: 13-17° (+1°); duration: 12 minutes; active milling patterns: upper.

NOTE: The rough milling steps (5.3.6) are carried out sequentially for each lamella. In contrast, the fine milling step (5.3.7) does not directly follow the rough milling, but fine milling steps are carried out sequentially for all the lamellae at the end of the session to minimize the hydrocarbon contamination on the lamella surface. An extra +1° stage tilt is used during fine milling step to increase the uniformity of the lamella thickness across its length.

6. Transfer of *Saccharomyces cerevisiae* lamella to cryo-TEM

6.1. Prepare a properly dried Dewar and fill it with LN_2.

6.2. Unload the samples with lamellae from the FIB/SEM microscope under cryo-conditions, transfer them to a grid box, and store them in a LN_2 storage Dewar for long-term storage. Alternatively, load the grids directly into cryo-TEM.

6.3. Correct orientation of the lamella relative to the cryo-TEM stage tilt axis is important (see accompanying video at 8:10 for more details). Ensure that the milling direction of prepared lamellae is perpendicular to the cryo-TEM stage tilt axis.

6.4. Pre-tilt the cryo-TEM stage to compensate for the lamella tilt relative to the grid plane and collect the tilt-series using dose-symmetric scheme[19].

NOTE: The magnitude of the pre-tilt (typically 6–8°) is determined by the angle between the TEM grid plane and the FIB direction during micromachining. The position of the lamella front edge in the image in cryo-TEM can be used to determine the sign of the pre-tilt angle. For that, the sense of the microscope stage rotation must be known. In our experimental setup, the position of the front edge of the lamella on the right side of the image taken in nanoprobe SA mode corresponds to negative pre-tilt.

REPRESENTATIVE RESULTS:

The *Saccharomyces cerevisiae* culture was harvested in the middle of the exponential growth phase. We prepared two types of specimens in which the cells were either distributed as small clusters of several cells over the surface of the TEM grid (**Figure 1A,C**) or formed a continuous monolayer over individual grid squares of the TEM grid (**Figure 1B,D**). The discriminative factor for the preparation of the sample with either distinct cell islets or the cellular monolayer is the concentration of the cell culture applied to the TEM grid. The harvested cell culture was concentrated to OD_{600} = 1.0 for the former case, or to OD_{600} = 30 to 60 for the latter case,

respectively. The sample for the preparation of the cellular monolayer was further supplemented with 5% v/v glycerol prior to vitrification. The glycerol is critical for the vitrification of the buffer solution, which fills the space in between the cells (**Figure 2**) as the reflections from the crystalline buffer can be detrimental for proper positional tracking and focusing during cryo-ET data collection.

In addition, the yeast suspension culture was blotted against the non-absorbent material such as the PTFE blotting pad or the custom 3D printed pad made of FlexFill 98A material. The blotting paper was positioned only on the backside of the grid with respect to the sample application (back-blotting). The back-blotting strategy is recommended for the suspension culture plunge freezing as blotting with the filter paper from both sides results in adhesion of the cells to the blotting paper (**Figure 1E**).

The protocol described here utilizes TEM grids clipped in the AutoGrid, which forms stable support for the grid and facilitates sample handling of the sample after the vitrification. This enforces a necessity that other sample holders and shuttles in FIB/SEM and TEM microscope can accept such a grid cartridge.

Upon transfer of the sample into the the FIB/SEM microscope, the specimen was first coated with a 0.3–1.0 µm layer of methylcyclopentadienyl platinum using the microscope gas injection system (GIS). An additional layer of the inorganic iridium was sputtered on the sample surface to harden the GIS layer and render the surface conductive. The lamellae were milled in multiple steps (**Figure 3**) where (I) the milling current, (II) the lamella width, and (III) the distance of the milling area above and below the samples were decreased in a stepwise manner. The final milling step ("polishing") was carried out at low current (10-30 pA) only from the top side of the lamella and with the sample inclined by an additional 1° towards the Ga+ beam. Utilization of the described protocol has on average resulted in 8-10 lamellae prepared on two TEM grids within one 6-8 hour session.

The TEM grids with the lamellae were subsequently transferred into a transmission electron microscope. The lamellae were first screened and only those which showed minimal curtaining (artefacts stemming from uneven milling across the lamella surface), low surface contamination level, and good cellular contrast (usually observed for lamellae with <200 nm thickness) were selected for the acquisition of the cryo-ET data. In addition, lamellae containing cracks across the whole length were discarded from data collection. In general, about 50% of the lamellae transferred to TEM were suitable for data acquisition. Tilt series were collected on the post-GIF K2 direct electron detector with the energy-selecting slit set to 20 eV. The data collection was carried out in SerialEM software[18] and the tilt series were collected using a dose symmetric scheme[19] with the tilt range of ±60° and the increment of 3°. The data was acquired at the magnification corresponding to the pixel size of 3.47 Å/px. The overall dose of 65 e/Å^2 was uniformly distributed over the individual sub-frames. The tilt images were collected as a set of three frames, which were subsequently corrected for the motion and radiation damage during data acquisition using MotionCor2[20] program. Parameters of the contrast transfer function were estimated using Ctffind4[21]. The tilt series were processed in eTomo[18]. The patch tracking routine

was used to align the images. The tomogram was reconstructed using a weighted back projection algorithm after 2x binning of the images, and subsequently filtered using SIRT-like filter (set to 8 iterations) in IMOD[18]. The tomogram segmentation was carried out manually in Amira software[22]. The reconstructed tomograms provide a high-resolution representation of the yeast cellular interior and enable us to observe organelles such as vacuoles or mitochondria at a high level of detail or study macromolecular complexes such as microtubules, or nuclear pore complexes *in situ* and under near-native conditions (**Figure 4**).

FIGURE AND TABLE LEGENDS:

Figure 1: FIB and SEM images of vitrified *S. cerevisiae*

FIB (**A**) and SEM (**C**) images of the small yeast clusters vitrified on the TEM grid. FIB (**B**) and SEM (**D**) images of the yeast forming a continuous monolayer on the grid surface. The sample was coated with GIS and Irridium layer before imaging. The scale bars in panels A-B corresponds to 10 µm. The yeast sample is blotted against non-absorbent material such as PTFE or FlexFill 98A (green) and with the blotting paper positioned from the backside of the grid (white, **E**).

Figure 2: *S. cerevisiae* lamellae

A TEM image of a lamella micromachined from the sample with continuous monolayer yeast over the grid surface. The reflections observed between the cells contained improperly vitrified medium/buffer (**A**, highlighted with red circles). A TEM image of lamella generated on the yeast vitrified into a continuous monolayer with the addition of 5% glycerol into the medium/buffer (**B**). Scale bar corresponds to 2 µm.

Figure 3: Cryo-FIBM workflow

Schematic depiction of the lamella milling process. The initial rough milling steps are performed at high FIB currents from both sides of the tentative lamella position (highlighted in green) whereas the final polishing step is performed only from the top side and at low FIB current (highlighted in orange, see accompanying video at 6:33).

Figure 4: Yeast organelles and macromolecular complexes depicted by cryo-ET

Slices of the reconstructed tomograms depicting a vacuole (**A**, scale bar: 200 nm), ribosomes (B, scale bar: 200 nm), a paracrystalline core of peroxisome (**C**, scale bar: 100 nm), microtubule (white arrow) in the proximity of unidentified fibrous structure (black arrow, **D**, scale bar: 100 nm), details of multiple microtubules (**E**, scale bar: 50 nm), a nuclear membrane with pores indicated by arrows (**F**, scale bar 200nm), mitochondrion (**G,H**, scale bar: 100 nm, the arrows indicate individual cristae), a bundle of unidentified filamentous structures (**I**, scale bar: 100 nm). Panels **B, C, D, E, G** contain a section of tomograms prepared from small clusters of cells whereas the sections of tomograms collected on lamellae from a monolayer of the cells are shown in panels **A, F, H, I**.

DISCUSSION:

The preparation of the cellular samples for cryo-ET is a complex workflow that requires utilization of several high-end instruments. The sample quality can be compromised during each

preparation step that influences the throughput of the whole protocol. In addition, the necessity of the sample transfer between individual instruments poses an additional risk of sample contamination or devitrification. Therefore, optimization of individual steps in the sample preparation workflow is of high importance to increase the throughput and reproducibility of the lamella preparation workflow. The protocol presented here describes the optimized preparation of *Saccharomyces cerevisiae* for structural characterization of macromolecular complexes *in situ* by cryo-ET.

The protocol describes the preparation of two types of yeast specimens that mainly differ in the concentration of the cells on the TEM grid. Both yeast samples yielded high-quality lamellae for cryo-ET and selection of the specimen type can be made in accordance with the goals of the particular study. The yeast forms isolated clusters of few cells randomly scattered over the grid surface in the first case, whereas a continuous monolayer of cells is present on the TEM grid surface for the second sample type. The former is suitable for fast lamella preparation thanks to the small volume of the material that must be milled away. The final lamella is fairly short, and, therefore, contains only 2-4 cellular cross-sections. The areas suitable for sample preparation are randomly distributed over the grid surface including the grid squares, which may partially restrict the automation of the lamella preparation workflow. The latter type of the specimen requires utilization of larger currents during the initial milling phase to retain the overall milling time. In addition, this type of sample is more prone to artifacts that stem from uneven milling (curtaining). Therefore, GIS is sputtered onto the sample surface for a 50% longer period than in the case of the sample with small cellular clusters to form a thicker protective layer. Next, the sample is sputtered with an additional layer of Iridium (alternatively platinum or gold) to cure the GIS layer, render it stiffer, and increase the sample surface conductivity. FIBM of additional areas on each side of the lamella (~2-5 µm from the lamella edge) during the first step of the rough lamella milling was found beneficial to diminish the number of broken lamellae most probably due to decreased tension in the final cross-section[23]. The final lamella is long and contains ~10 cellular cross-sections, which increases the number of regions suitable for cryo-ET. The improper vitrification of the medium or buffer between the cells can be easily attenuated by the addition of the cryo-protectant to the buffer solution (5% glycerol used in this study). Since most of the squares are suitable for lamella preparation, the sample with cells organized into a continuous monolayer is well suited for unsupervised lamella preparation.

Another important aspect in the lamella preparation workflow is the transfer to the transmission electron microscope and proper positioning of the lamella to the microscope stage tilt axis. Optimally, the lamella main axis is perpendicular to the tilt axis of the microscope which affords tracking and focusing at the height of the imaged region and prevents the lamella edges from shielding the field of view at high tilt angles. When collecting the cryo-ET data using the dose-symmetric scheme,[18] the sample should be initially rotated in the microscope to compensate for the tilt of the lamella with respect to the grid plane.

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Instruct-ERIC Centre (LM2018127). We acknowledge the support obtained from Thermo Fisher Scientific Brno.

DISCLOSURES:

The authors have nothing to disclose.

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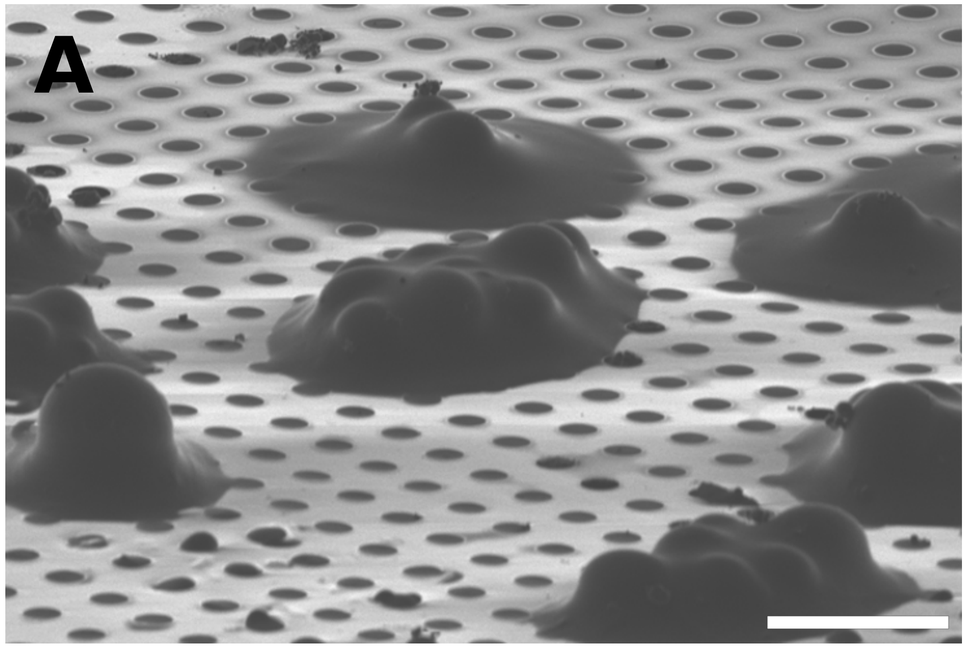

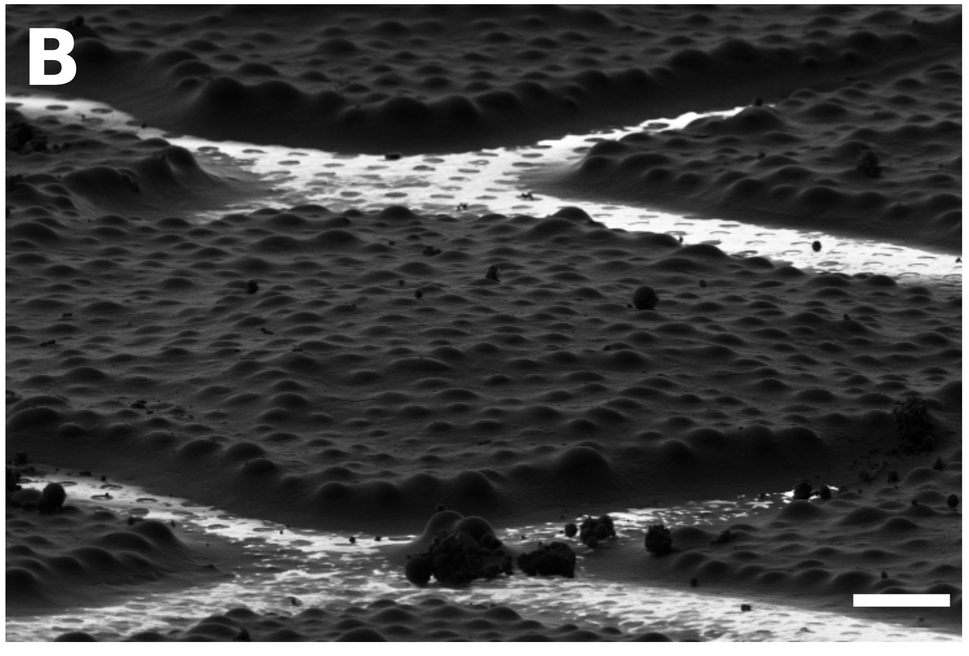

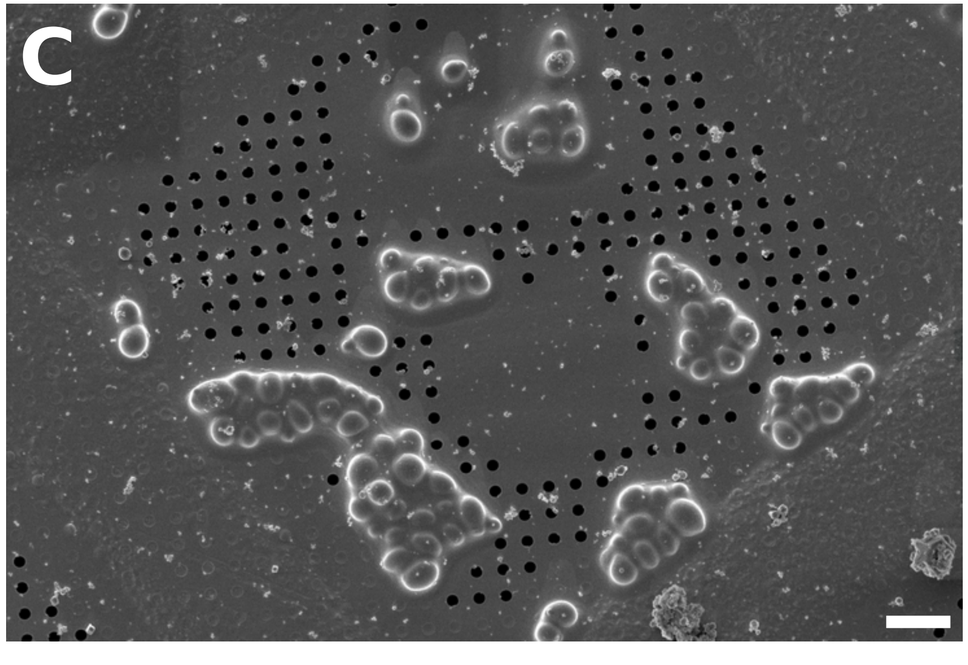

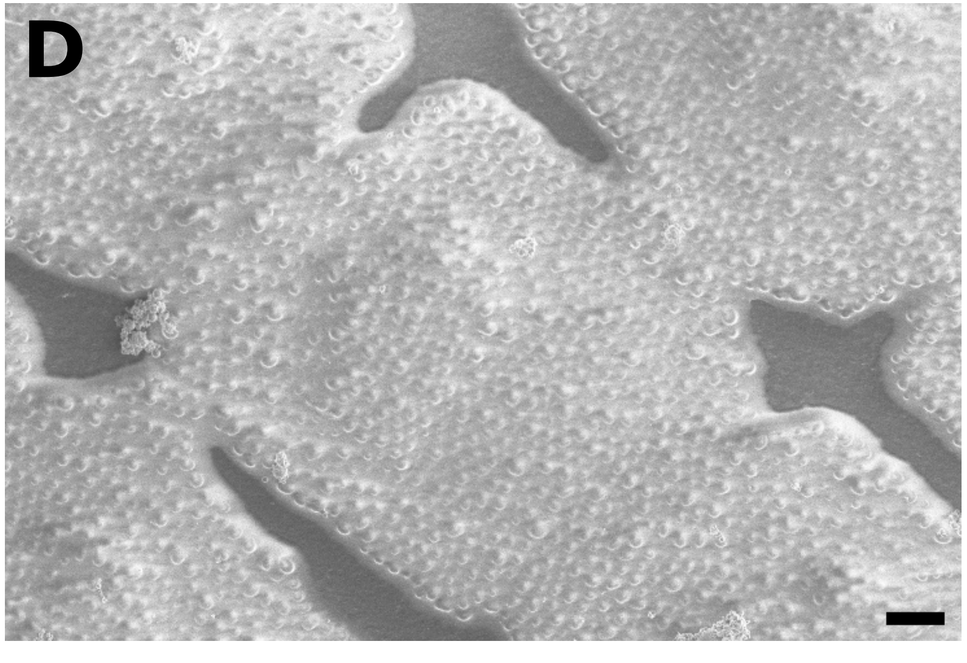

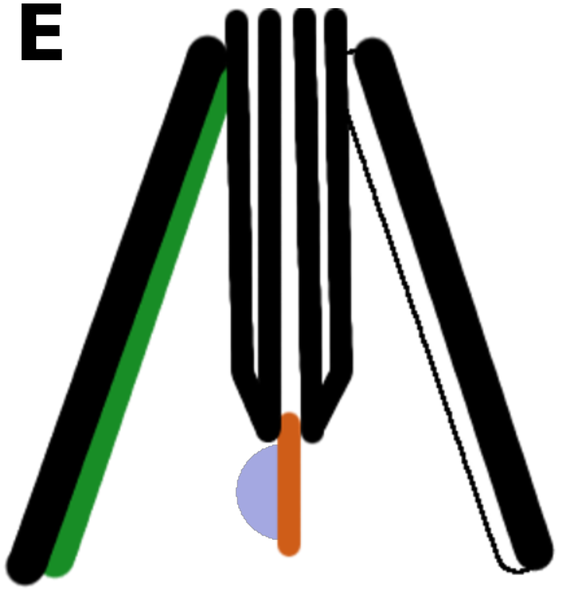

Figure

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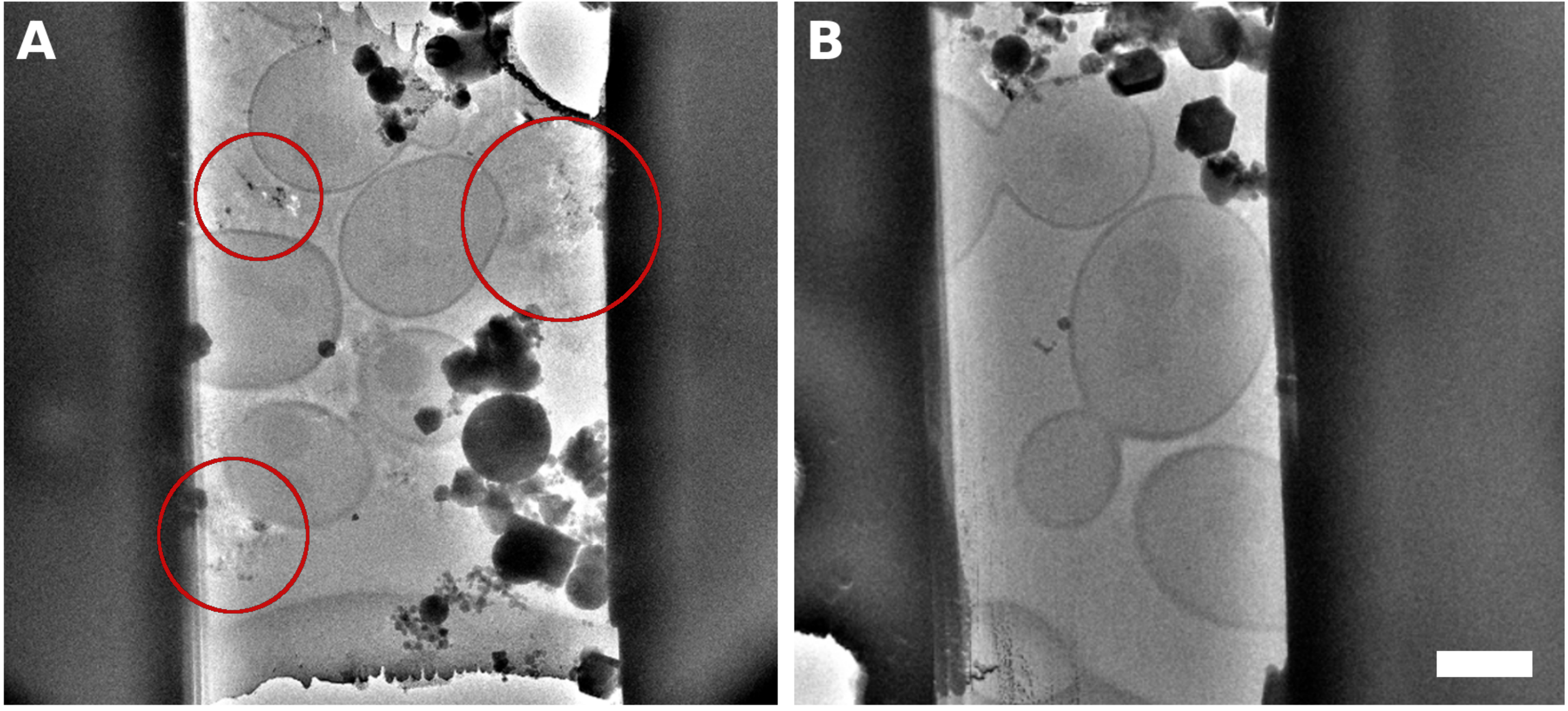
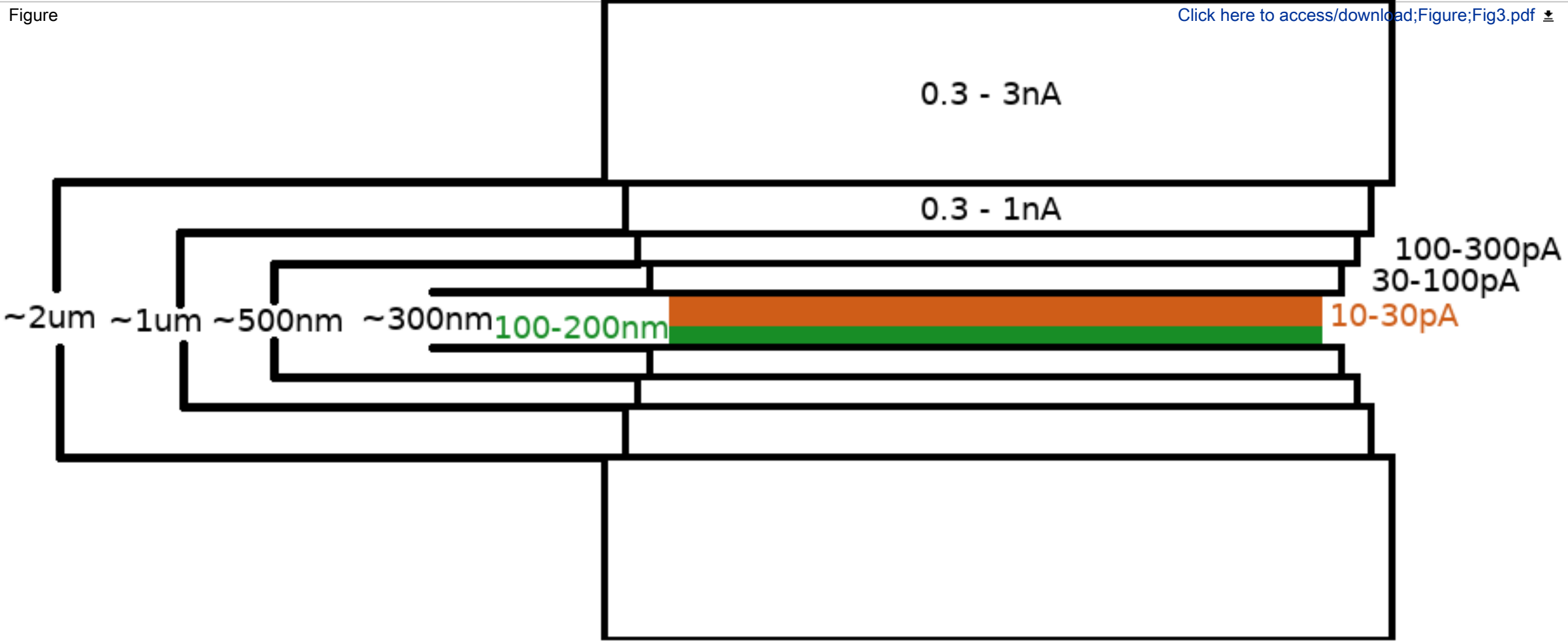


Figure



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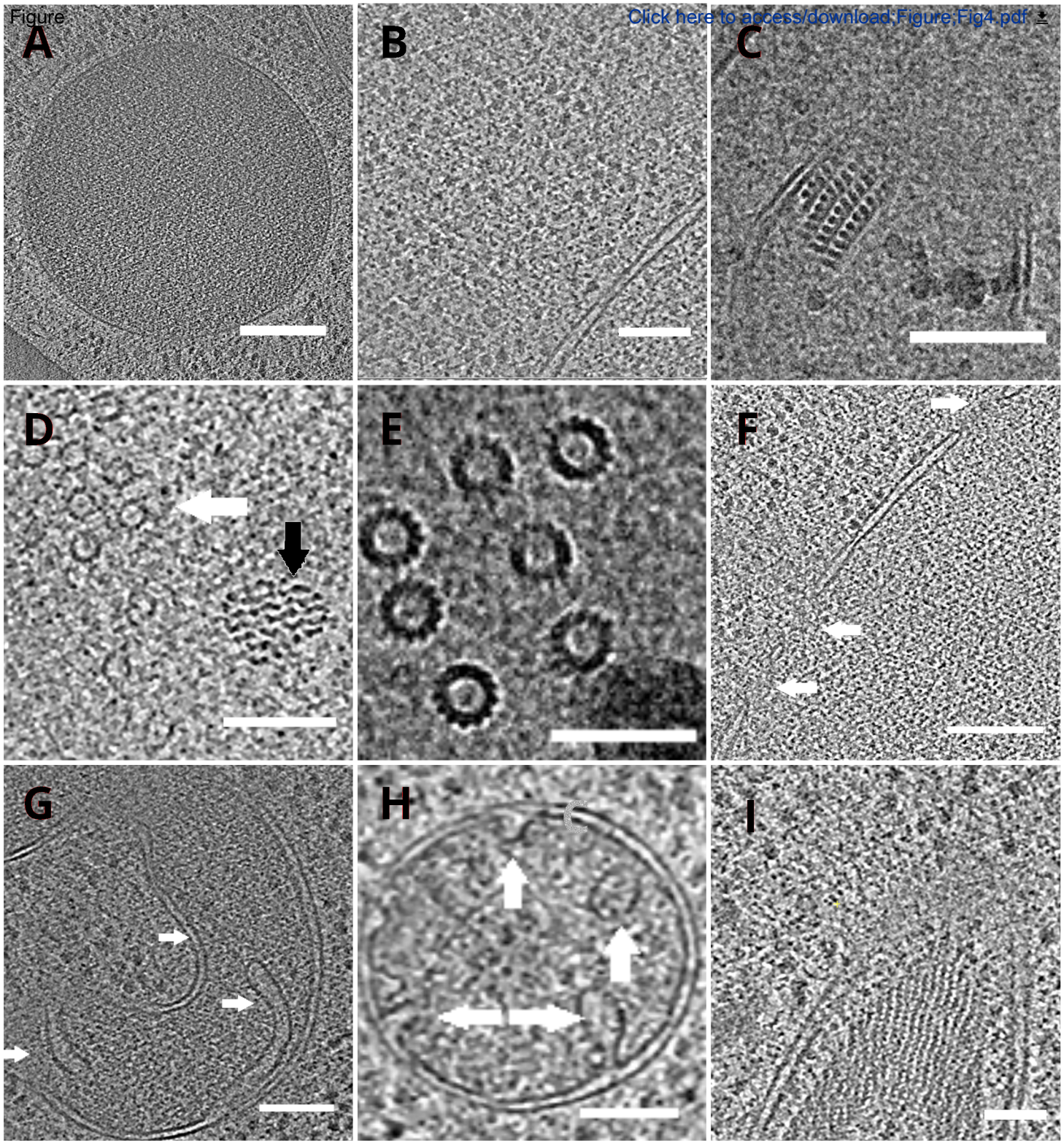

Dear Editor,

please find below our responses to the questions raised during review of our manuscript. I hope we have managed to appropriately address all the question raised during revision. Please do not hesitate to contact me in case additional questions appear during the review of the revised version.

Thank you.

Kind regards,

Jiri Novacek

Changes to be made regarding the video:
1. Composition
The resolution is 1024x576, which is too low. The resolution needs to be 1920x1080.

Response: The video has been formated to 1920x1080 resolution.

The text and images are too close to the edge of the frame for all the slides noted in the timecode. Please resize the text and images so there's more room around the border. Instead of resizing the slide so there's no black bars on the side, make the background white so those bars are no longer visable:
00:04 - 00:15, 02:17, 06:13, 07:02, 08:17

Response: We have rearranged the slides to get more space around the edges. However, we are constrained to transparent background by the video editor. We are convinced that the text is well visible under these conditions.

03:02 There's a weird glitch where the left side of the next video appears while stil showing the right side of the previous clip, then after a second the full clip appears. Please fix.

Response: We have fixed the glitch.

07:32 It looks like there's a stabilizing effect on this clip because I can see the clip rotate at the bottom where you can see the edge a bit. Please remove this effect.

Response: The issues has been removed.

2. Pacing
"00:45 - 00:51 Please shorten this shot by removing the cap being placed back on the tube, and fade from the pipette in the tube, to the pipette going into the vial. This will also shorten the gap of no narration afterwards.

Response: We have shortened the corresponding part.

02:32 - 02:38 This shot goes on too long without the narration. Please cross dissolve to the pipette right after the liquid nitrogen is being poured in.

Response: We have shortened this part.

02:53 - 03:02 Please cross dissolve to the part where the ring goes in the machine much quicker. It goes on for too long before anything happens and there's no narration during that part.

Response: We have shortened the part of the video in question.

04:51 There's a weird dip transition here, please remove this."

Response: The transition has been corrected.

Please upload a revised high-resolution video here:
https://www.dropbox.com/request/zHm7CEO2H9tW65q8s9rP?oref=e

Reviewers' comments:

Reviewer #1:

Major Concerns:
The two types of sample elaborately discussed in the written manuscript are not mentioned in the sample preparation of culture or in plunging sections in the video.

Minors Concerns:
Figure 1: scale bars still missing in A-D. should be corrected

Response: Scale bars were added to Figure 1 and the figure legend was updated accordingly.

Line 37: "think layer" should be corrected to "thin layer"

Response: The typo has been corrected.

Line 55: "context" used twice int he same sentence

Response: The corresponding sentence has been changed to remove the repetition.

Line 62: "structure" used twice in the same sentence

Response: The corresponding sentence has been changed to remove the repetition.

Line 70: please correct "for the in vitrified biological specimens "

Response: The typo has been corrected.

Line 139: "to protect drying " add from drying

Response: The preposition has been included as suggested.

Line 217: "1.7.6. Mix cell suspension with 5% glycerol shortly before vitrification." it is not clear if 5% represents the final concentration in the solution

Response: The sentence has been changed to clarify the meaning.

Video:
1:24 - the sentence is incomplete in the first section, and repeated again the following section.
Audio should be better edited

Response: We have corrected this issue in the video.

1:44 - It should be specified at this point the two types of sample to be prepared (OD 1 / 30). it is also not clarified at which point Glycerol would be added to the culture.

Response: We have included a note on the addition of glycerol (1:40 in the video).

2:47 - Phrase gets cut off in the middle

Response: We have corrected this part of the video.

3:55 - AutoGrid name should be changed to grid cartridge

Response: We have changed the text in the video as requested.

5:01 - more specific description is need: the carbon side with cell should face upwards, coinciding with the flat side of the grid cartridge. Most lab make use of special FIB grid cartridges with a cut-out to allow show angle milling with the FIB. It shoud be noted in the video that if using such cartridges, the cut-out of the grid cartridge should be carefully aligned at 12 O'Clock.

Response: Even though the cryo-FIB cartridge is not used in our protocol, we have added the requested note to the video (4:46).

6:26 - Phrase gets cut off in the middle

Response: We have corrected this part of video and removed the issues with audio.

7:06 - image at bottom left corresponds to OD=30 (cell monolayer). Authors should place the correct image to represent the morphology of the sample at OD=1 at this position, and move the current image to bottom right to accurately represent the sample type. alternatively, it should be properly annotated. the range Gycerol to be used is very broad. should be more specific.

Response: A slide showing lamella milled either in a cell cluster, or cell monolayer was rearranged according to the suggestion.

Reviewer #4:
Manuscript Summary:
Moravcová et al. provide a detailed description of yeast cell culture, preparation for vitrification, and sample processing using FIB milling for cryo-electron tomography. Their protocol is well written, easy to follow, and describes two related procedures (suspended cells and monolayers), which adds to the impact and (hopeful) uptake of this method. The accompanying video is clear and adds to the clarity of the paper. If possible, refer to specific timepoints in the video within the text.

Major Concerns:
None

Minor Concerns:
Below I have appended a few very minor suggestions, but I fully recommend publication of this method.
line 67 - state temperature of LN2

Response: The liquid nitrogen temperature has been added to the text as requested.

Line 146 - Wording is ambiguous - suggest:
"We have observed that the exponential phase is reached after ~15 h in solution when colonies are picked from agar plates that have been cultured at ambient temperature for 4 weeks. See also Note 3 below."

Response: We have changed the sentence as suggested.

Line 191 - You state "Prepare S. cerevisiae cell culture according to the protocol in the section Cultivation of Saccharomyces cerevisiae suspension cell culture" > Do you mean section 1.3. Culture Saccharomyces cerevisiae in suspension?

Response: The sentence has been corrected to precisely correspond to appropriate section in the protocol and the reference to the section number has been included.

Line 226 - Which device? From the text below I'm guessing a Vitrobot but good to be clear.

Response: The term "plunge freezing device" was replaced by "vitrification robot" as we were specifically asked by the Editor to remove all the reference to specific products from the protocol.

Line 468 - "the specimen was first coated with a 0.3-1.0 µm layer of methylcyclopentadienyl platinium" - was this performed before imaging in Fig 1? Otherwise, reword to make clear it was coated before milling, not immediately upon insertion.

Response: Yes, the GIS and Irridium coating was carried out before imaging shown in Fig. 1.

Line 481 - Explain the term "curtaining"

Response: Explanation of the term has been added into the text.

Figure 1 figure legend - Have these been coated with metal yet? State one way or the other
Explain colours. E.g., "(E) The yeast sample is blotted against non-absorbent material such as PTFE or FlexFill 98A (green) and with the blotting paper positioned from the backside of the grid (white)."

Response: The figure legend has been changed to clarify that the images were taken after metal coating and the description of panel E has been changed as suggested.

Figure 2 - there are no red circles in my version

Response: The Figure 2 was re-uploaded during re-submission. The panel A contains three red circles.

Figure 3 legend - Explain orange and green text. Maybe refer to video timepoint for more info.

Response: The figure legend has been updated to explain more clearly the meaning of the orange and green colours. A reference to the time point in the video corresponding to the polishing step was included.

Figure 4 - Explain arrows - e.g., "Slices of the reconstructed tomograms depicting a vacuole (A,

scale bar: 200 nm), ribosomes (B, scale bar: 200 nm), a paracrystalline core of peroxisome (C, scale bar: 100 nm), microtubule (white arrow) in the proximity of unidentified fibrous structure (arrow of another colour) (D, scale bar: 100 nm), details of multiple microtubules (E, scale bar: 50 nm), a nuclear membrane with pores indicated by arrows (F, scale bar 200nm), mitochondrion (G,H, scale bar: 100 nm), a bundle of unidentified filamentous structures (I, scale bar: 100 nm). Panels B, C, D, E, G contain a section of tomograms prepared from small clusters of cells whereas the sections of tomograms collected on lamellae from a monolayer of the cells are shown in panels A, F, H, I." What are the arrows pointing at in Fig 4G and H?

Response: The figure legend has been updated as suggested. The arrows in panels G and H point to longitudinal and transverse view of mitochondrial cristae.

Line 541 - replace "lead" with "yielded"

Response: The sentence has been changes as suggested.

Line 546 - "The final lamella is fairly short" - why is it shorter than monolayer?

Response: The lamella is milled through a region which might be only one wide in the milling direction in the case of small clusters. As a result, the lamella can be very short in this case. However, this is never the case in the sample comprising yeast monolayer where the cells are close one to each other and the lamella is always milled across multiple cells.

Reviewer #5:

Manuscript Summary:

This manuscript from Jiri Novacek and colleagues present a detailed protocol about how to prepare yeast cells for cryo-ET analysis. The cryo-FIB is the key for milling the sample of yeast cells to a reasonable thickness. The authors introduced 2 types of sample preparations including isolated yeast cell patches and continuous monolayer. The overall writing is straightforward and the attached video is clear.

Major Concerns:

I have the main concern about reference and citation:

You can not neglect the paper published in Nature protocol 2020 Jun;15(6):2041-2070. Tilted as preparing samples from whole-cell using focused-ion-beam milling for cryo-electron tomography.

Response: The corresponding reference was included into the manuscript in order to properly acknowledge the work of others.

Line 509(R2_reversion), tomogram was reconstruted by etomo, you need to add references about software, reconstruction method and etc.

Response: More details about the procedure used for tomogram reconstruction was included and corresponding reference to IMOD was added.

Line 513, in your attached video, you made a segmentation. Thus, you need to add a description of segmentation plus relative reference.

Response: The tomograms were segmented in Amira. The information was added to the text together with the corresponding reference.

Minor Concerns:
Line 56, delete when combined,

Response: The text was deleted as suggested.

Line 81, the description is not accurate. No paper suggests the cryo-FIB generates input for high-resolution cryo-ET. Reference 7 you cited has shown the highest resolution structure of in situ ribosomes. This amazing result can be attributed to specific samples and advanced software.

Response: The corresponding sentence has been changed to clarify the meaning of the text.

Line 91, similar to Escherichia coli well studied as a prokaryotic model organism in bacteriology.
Line 100, This protocol
Line 131 and Line 151, need switch sequence
Line 217, 5% is a final concentration? How to prepare in this step in detail. Because adding additional glycerol will change the concentration of yeast cells in the solution.
Line 225, (carbon film side facing up)
Line 240, mount the tweezer
Line 248, transfer the grid from ethane to LN2.

Response: The text of the manuscript has been changed in accordance with the suggestions.

Line 255, consist of cryoFIB autogrid, TEM grids and C-clip ring

Response: The cryo-FIB autogrid has not been used in this case. We prefer to keep the term C-ring instead of the autogrid as C-ring is the name of the product when purchasing from the producer.

Line 309, you might add a note about autogrid direction.

Response: There is no specific direction as the cryo-FIB autogrid has not been utilized in this protocol.

Line 318-321, the description is not accurate. Should be optional. On the contrary, the charging caused by SEM would be helpful for discriminating the density between the yeast cells and background.

Response: We have changed the manuscript and denoted this section optional as requested. However, we always sputter coat the sample prior milling. In our hands, the charging effect are only helpful when imaging surface of the lamella after milling.

Line 351, you need to add detailed information about adjusting eucentric height during tilting the stage from 45 to 15 degrees.

Response: The eucetric height has to be set for each milled region separately. This in described in section 5.3.1. We have modified a Note related to this section to comprise the detailed description of how to set a eucentric height for a particular position.

Line 354-384, they all are belonging to checking the quality of grids. Could you merge all together in a simple and concise way?

Response: We prefer to keep the structure of the quality control as is written in the protocol. We have found this more structured description instructive for the novice users we trained in our lab.

Line 389, you would demonstrate clearly that you do rough milling on several targets, then when the rough milling on all pre-selected targets complete, you perform fine-milling one by one in a short time.

Response: The note following the section 5.3.7.1 has been updated to clarify that the fine-milling (polishing) step as carried out on all the lamellae at the end of the session.

Line 450, would be better if you provide an example, e.g 16 degrees.

Response: Typical number for the stage pre-tilt has been added to the manuscript.

Line 475, replace preferred with recommended.
Line 501, the tilt series were collected
Line 505, angstrom unit
Line 506, sub-frames
Line 508 estimated and determined
Line 509, the CTF-corrected images were aligned

Response: The text of the manuscript has been changed in accordance with the suggestions.

Line 510, how do you generate tomogram by sirt or wbp in a binning factor?

Response: The tomograms were reconstructed from 2x binned data using the weighted back-projection algorithm. The text has been changed to contain this information.

Line 511, segmentation description.

Response: The tomograms were manually segmented in Amira software.