Waffle Method: A general and flexible approach for FIB-milling small and anisotropically oriented samples

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Abstract

Cryo-FIB/SEM has emerged from within the field of cryo-EM as the method for obtaining the highest resolution structural information of complex biological samples *in-situ* in native and non-native environments. However, challenges remain in conventional cryo-FIB/SEM workflows, including milling specimens with preferred orientation, low-throughput when milling small and/or low concentration specimens, cellular specimens that concentrate poorly in grid squares, and thick specimens that do not vitrify well. Here we present a general approach we call the 'Waffle Method' which leverages high-pressure freezing to address these challenges. We illustrate the mitigation of these challenges by applying the Waffle Method to reveal the macrostructure of the polar tube in microsporidian spores in multiple complementary orientations by cryo-ET, which was previously not possible due to preferred orientation of the spores on the grid. We then apply the Waffle Method to several more biological samples and to different cryo-FIB milling hardware, including automated milling, to show broadness of method. We also present a unique and critical stress-relief gap design specifically for waffled lamellae. Additionally, we describe applications of the Waffle Method which are currently being explored, including the possibility that the Waffle Method may be used to resolve otherwise intractable single particle cryo-EM samples. We propose the Waffle Method as a way to achieve many of the advantages of cryo-liftout on the specimen grid while avoiding the long, technically-demanding process that cryo-liftout requires.

Introduction

Conventional cellular cryo-focused ion beam milling scanning electron microscopy (cryo-FIB/SEM)[1] is developing as a fruitful method for structural studies of cells thinner than about 10 µm. In some instances, cryo-FIB/SEM followed by cryo-electron tomography (cryo-ET) and sub-tomogram processing has produced *in-situ* 3D structures of molecular complexes at resolutions on the order of 1 nm[2–6]. Methods for promoting cell adhesion onto grids have been developed[7–9]. However, the broad application of conventional cryo-FIB/SEM with reasonable throughput is often challenged by specimens with dimensions smaller than about 2 µm and which adopt a preferred orientation on the grid.

Three previous approaches have been used to address issues of specimens thicker than about 50 µm prior to FIB-milling lamellae for imaging in a transmission electron microscope (TEM). Cryo-ultramicrotomy has been used to thin vitrified tissues from hundreds of microns thick to 20 – 30 µm prior to conventional cryo-FIB wedge milling[10]. Recently, cryo-microtomy and cryo-FIB-mill trenching of vitrified *C. elegans in-situ* followed by micromanipulator cryo-liftout and further FIB-milling on custom EM grids has been demonstrated[11,12]. Yet few labs possess both the equipment and expertise to carry out such studies. An alternative method for obtaining three-dimensional information from native specimens *in-situ* is automated cryo-slice-and-view (cryo-ASV). Cryo-ASV is performed by using a high-pressure freezer (HPF) to vitrify a block of specimen, which is then inserted into a cryo-FIB/SEM for sample manipulation and imaging. The bulk specimen is then thinned down until an area of interest is identified. The surface is then iteratively imaged and milled, allowing for the whole volume to be imaged at resolutions of several tens of nanometers in (x,y,z), a resolution which is often a restrictive limit for molecular studies[12].

Here we present a general method, called the 'Waffle Method', for cryo-FIB milling lamella of a broader range of specimens *in-situ* compared to conventional cryo-FIB/SEM. The Waffle Method has the following advantages that we substantiate herein: (1) there are far fewer restrictions on the size of the specimens that can be reliably milled (from nanometers to tens of microns); (2) preferred orientation issues for specimens within this size range are resolved; and (3) several very large (between 10 x 10 µm and 70 x 25 µm in x,y) and specimen-dense lamellae may be prepared regardless of the specimen size, which substantially increases throughput. Moreover, the Waffle Method inherently solves vitrification issues of all sample types

through the use of an HPF, does not require additional FIB/SEM hardware for preparation, and may be used to FIB-mill specimens with thicknesses up to about 50 µm. In developing the Waffle Method, a modified form of gap milling for lamellae stress-relief called notch milling proved to be critical. We describe notch milling and its potential mechanism herein.

To exemplify the advantages of the Waffle Method, we present a comparison of the Waffle Method and conventional cryo-FIB/SEM on native, intact microsporidian spores. Microsporidia are obligate, unicellular, spore-forming parasites that possess a ~100 µm long polar tube to infect target host cells[13,14]. The object of interest within the dormant microsporidian spores - the polar tube - is in a fixed orientation relative to the spore[14] and the spores exhibit preferred orientation on the grid, constituting an intractable orientation issue for conventional cryo-FIB/SEM. We show that the Waffle Method solves the preferred orientation issue of the polar tube within the spores while retaining nanometer-level features. Moreover, the Waffle Method allows for more efficient and higher-throughput FIB-milling of these spores compared to conventional cryo-FIB/SEM due to the significantly larger lamella that are created, which is a generalizable advantage for all specimens of comparable size. To illustrate the broadness of the Waffle Method, we apply it to three additional cell types - yeast *S. cerevisiae* cells, *E. coli* cells, and HEK 293S cells - and to different cryo-FIB/SEM hardware, namely the Aquilos 2 combined with automated lamellae milling.

The Waffle Method may also have the following additional advantages that are currently being explored and which will be discussed here: (1) thin complexes *in vitro* with lengths on the order of micrometers to millimeters may be studied structurally from all directions, such as filaments and microtubules; (2) proteins in bulk solution may be studied by single particle cryo-electron microscopy (cryo-EM) or cryo-ET, which may be the only available method amenable to samples that do not behave in the thin ice of conventional cryo-EM grids due to air-water interface issues causing denaturation, aggregation, preferred orientation, etc.[15–17]; (3) very long and skinny cells may be studied more completely; (4) cells may be studied that are difficult to mill conventionally due to suboptimal affinity to grids, suboptimal concentration in grid squares, wicking/hydration issues on grids, etc.; (5) tissues and multi-cellular organisms may be studied as an alternative to the cryo-FIB/SEM liftout method.

The initial developer of the Waffle Method, Kotaro Kelley, unfortunately passed away before the method could be reported on in the literature. Kotaro conceived of the Waffle Method, beginning in 2018, as a specific solution to several collaborative projects, which he quickly realized was a general solution to a broad range of issues in the field of cryo-FIB/SEM. Kotaro's

work from 2018 to 2019 was published as the first version of this preprint, followed by our re-discovery of his method in this version. Kotaro spent countless nights and weekends of trial and error developing the Waffle Method. Here we report on the Waffle Method in lieu of Kotaro to recognize his work and honor his dedication. We also report on Kotaro's independent exploration of stress-relief gaps for lamella. This manuscript serves as a time capsule of his work in these areas.

Results

Overall workflow

Figure 1 and Supplemental Video 1 show the overall, general Waffle Method workflow. Workflows for each specific sample are detailed in the Methods. Before making the waffle, the hardware needs to be prepared (Step 0): (a) planchette hats should be polished - first with varying grit sandpaper, then with metal polish (Supplemental Figure 1) - to reduce lamellae curtaining; (b) 1-hexadecene is applied to the planchette hats and the optional spacer ring; (c) the EM grid is rigidified by applying ~25 nm layer of carbon to the non-grid bar side of the grid; (d) ethanol is used to clean the HPF tip; and (e) the EM grid is glow discharged/plasma cleaned.

Waffle making (Step 1) is performed as follows: (f) the sample and the planchette hats are assembled into a waffle form with the sample placed on the grid bar side of the EM grid as shown in Supplemental Figure 2 and Supplemental Videos 1 and 2; (g) the sample is high-pressure frozen; and (h) the planchette hats are removed, the grid is carefully clipped into an autogrid, and an optional conductive platinum coat is sputtered on. At this point the waffle grid may optionally be screened in a cryo-FLM.

The waffle grid is then transferred to a flat cryo-FIB/SEM grid holder (or tilted grid holder if near-orthogonal milling to the grid is possible) and then to the cryo-FIB/SEM chamber to mill the waffle (Step 2) as follows: (i) several microns of platinum are deposited using a Gas Injection System (GIS); (j) grid bar lines are drawn for convenience; (k) two trenches for each subsequent lamella are milled (>1nA at an angle of >45° to the plane of the grid) leaving about 30 µm of sample left in the slab between; and (l) the grid is transferred to a tilted cryo-FIB/SEM holder (if not already in one) to mill the lamellae. To protect the resulting lamellae, subsequent milling should always be performed with the ion beam intersecting a part of the slab with a leading edge of platinum GIS. At more shallow angles (~20° or less from the grid plane), the slab is thinned down stepwise to about 3 µm thick while reducing the ion beam current. With a similar current, a notch is milled completely through one side of the lamella to provide stress-relief (Figure 2). Current is reduced and the lamellae is milled down to about 1.5 µm thick while tilting the stage ±1° with a tab left beside the notch. Current is reduced further while milling the lamella down to about 500 nm thick while tilting the stage ±0.5°. The lamella is then polished down to the final desired thickness. Each stage in milling should be performed in parallel across each lamella after the lamellae are thinner than several microns. Parallel milling reduces

contamination, redeposition, and condensation build-up across all lamellae. Supplemental Figure 3 shows images during coarse and fine waffle milling. The finished lamellae are then sputtered with several nanometers of conductive platinum. The waffle grid with lamellae is then transferred to a TEM for cryo-ET collection followed by 3D analysis.

Hardware requirements and recommendations

The Waffle Method requires the following hardware equipment:

1) Planchette hat polishing equipment (sandpapers with multiple grits, fine-grain polishing solution, and Kimwipes).
2) 1-hexadecene.
3) At least 5 µL of sample for waffling.
4) A sputtering device for carbon evaporation at room temperature.
5) A high-pressure freezer (HPF) capable of vitrifying samples onto EM grids.
6) A cryo-transfer system, preferably with vacuum control between transfer steps.
7) A high-vacuum sputtering device for conductive platinum deposition at cryo-temperature.
8) A tilted cryo-FIB/SEM grid holder (the tilt angle should allow for shallow lamella milling).
9) A cryo-FIB/SEM.
10) A GIS system for platinum GIS coating.
11) Common cryo-EM equipment: carbon/gold EM grids, glow discharge cleaner or plasma cleaner, HPF planchette hats, tweezers, liquid nitrogen and dewars, cryo-gloves, eye protection (goggles/face mask), pipettes, and ethanol for cleaning.

We recommend the following additional equipment for the Waffle Method:

1) Humidity-controlled environment with humidity set to ~10% RH to minimize contamination.
2) 2-methylpentane.
3) EM grid spacer ring to adjust waffle thickness.
4) Magnifying glass and/or camera for loading grids in proper orientations.
5) A flat cryo-FIB/SEM grid holder for easier trench milling, if necessary.
6) A cryo-FLM system for screening waffles before/during milling and analyzing waffled lamellae after milling and/or after cryo-ET collection.
7) Fluorescently-tagged regions of interest in the specimen for FLM identification.

Waffle Method applied to small specimens with preferred orientation issues

To exemplify several of the advantages of the Waffle Method, we applied the method to dormant microsporidian *Encephalitozoon hellem* (*E. hellem*) spores. *E. hellem* spores are about 1.5 µm in diameter and 2.5 – 3.5 µm in length. Inside each unactivated microsporidian spore is a long, coiled polar tube with a diameter of ~130 nm, and several other organelles. Due to their shape, microsporidia on conventionally-prepared plunge-frozen EM grids almost exclusively lay on the grid with their long (anterior-posterior) axis parallel to the plane of the grid (Figure 3a). The polar tube, the most prominent organelle inside the microsporidian spore, is predominantly wound at a tilt around the long axis of the spore on the inside edge of the spore wall[14] (Figure 3e, schematic diagram). This co-dependence of polar tube orientation in the microsporidia and of the preferred orientation of the microsporidia on the grid, as shown in Figure 3a,b,e, results in intractable preferred orientation of the polar tube in lamellae after conventional cryo-FIB/SEM milling. Specifically, milling individual microsporidian spores inevitably leaves only axial views of the polar tube in the remaining lamella. Moreover, the throughput and accuracy of milling individual or small groups of microsporidia is considerably low (Figure 3a,b), and the resulting lamella are difficult to collect cryo-ET images due to the very small lamella and the location of the polar tube near the spore wall.

We applied the Waffle Method to microsporidia (Figure 3c-g) in order to visualize the polar tube in dormant spores, to solve the preferred orientation issue, to reliably mill these small cells, and to improve the throughput of cryo-FIB/SEM preparation and of cryo-ET collection while retaining nanometer-level features. The following workflow was used, which is depicted and shown in Figure 1 and Supplemental Video 1 & 2. Details of the specific Waffle Method workflow used here are presented in the Methods.

Prepare hardware and waffling the sample

To reduce the amount of curtaining that occurs during cryo-FIB-milling, the topography of the top of the waffle should be as uniformly smooth as possible above areas that will be milled (Figure 3c, Supplemental Figure 1). Deposited layers of conductive platinum and platinum GIS increase the thickness of the waffle, but do not change the topography substantially. To smooth the top waffle surface, we apply metal polish paste to each planchette hat using a Kimwipe and polish for several minutes per side until all visible features disappear.

To rigidify the grid, about 25 nm of carbon is sputtered onto the non-grid bar side of the grid. We found during waffle development that the survivability of grid squares during grid handling and high-pressure freezing is substantially increased if the grid has an additional layer of sputtered carbon. This is due to several steps in the Waffle Method imparting considerable forces onto the EM grid: The HPF process delivers a substantial impulse to the grid; the resulting waffle often has non-uniform coverage radially, causing differential stress and strain across the remaining open areas of the grid; and the several transfer steps that increase the risk of grid damage (waffle assembly, waffle disassembly, grid clipping, cryo-FIB/SEM holder loading/unloading, optional cryo-FLM loading/unloading, cryo-TEM loading).

The planchette hats and optional spacer ring are coated with 1-hexadecene to allow for easier disassembly of the waffle after freezing[18,19]. The HPF tip is cleaned with ethanol. The waffle grid is assembled in the HPF tip as shown in Supplemental Figure 2 and Supplemental Video 2: Several microliters of sample are applied to the grid bar side of the EM grid in the bottom planchette hat, the top planchette hat is placed on and excess sample is wicked away with a Kimwipe, and the HPF tip is closed. The planchette-waffle grid assembly is then transferred to the HPF where it is quickly high-pressure frozen. The waffled assembly is then removed from the HPF tip in LN2 before the planchette hats are disassembled to reveal the waffled grid.

The waffled grid is then transferred to the cryo-FIB/SEM prep chamber to sputter several nanometers of conductive platinum onto the grid, then to the cryo-FIB/SEM main chamber where several microns of platinum GIS is deposited onto the grid. Two trenches (several tens of microns in dimensions and apart) per area of interest are milled at angles as close to perpendicular to the grid plane as possible (Figure 3c). Partway through coarse milling, a notch is milled into one side of each lamellae (Figure 2 depicts the workflow). The slabs are then milled in parallel at an angle of 17° down to about 200 nm thick (Figure 3d shows a finished lamella). Several nanometers of conductive platinum are then sputtered onto the grid and lamellae before transferring the grid to a TEM for cryo-ET collection. Specific details of the milling steps are provided in the Methods.

Analysis of waffled microsporidian spore sample

Figure 3d shows a waffled microsporidian spore lamella in the SEM. The lamella is about 30 x 20 µm in size with a notch milled into the right-hand side. Several microns of platinum are still

present on the top of the notch while a micron or less are remaining on top of the finished lamella, illustrating the importance of coating the waffle with a sufficient amount of platinum to a sufficiently smooth waffle. Creating a sufficiently smooth waffle and applying a sufficient amount of platinum GIS is required for milling thin lamellae and minimizing curtaining (Supplemental Figure 4). In contrast to conventional plunge freezing and cryo-FIB/SEM (Figure 3a,b), the waffled lamella is replete with microsporidia that are in several orientations, which increases throughput during both cryo-FIB/SEM and cryo-ET (Figure 3d).

Figure 3d-g depicts how waffled lamellae of microsporidia solves the preferred orientation problem inherent with the specimen and the object of interest in the spores, the polar tube. The microsporidian spores in the waffled lamellae exist in several orientations, allowing for the full 3D structure of the polar tube to be visualized. The low-mag images in Figure 3e,f show TEM images of the spores, including an oblique-view in Figure 3f which is not present in conventionally prepared lamellae (Figure 3a). The schematic diagrams of the spores in Figure 3e,f depict cross-sections of approximate locations where tomograms were collected. The bottom images in Figure 3e,f show slice-throughs of 3D tomograms collected at these two locations. Roughly orthogonal orientations of the polar tubes are present in these tomograms (Supplemental Video 3), uniquely allowing for nanometer-level features of the polar tube to be visualized. Figure 3g shows 4x magnified views of the square dotted black line inset in the bottom images of Figure 3e,f. These magnified views show an axial view and a side view of polar tubes with bumps that are about 2.5 nm in dimension clearly visible on the second cylindrical layer of the tube.

Waffle Method applied to three additional cellular specimens

To illustrate the broadness of the Waffle Method, both in sample type and in workflow, we applied it to three additional cellular specimens: (1) yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) cells, (2) *E. coli* BL21 (DE3) cells infected with *Leviviridae* PP7-PP7 virus capsid proteins, and (3) HEK 293S GnTI^{-} suspension cells. Each specimen was prepared, waffled, and FIB-milled as described in the Methods.

Each of the four cellular samples presented in this work were propagated/cultured/expressed/harvested, centrifuged, then resuspended in solution prior to waffle making. Waffle grids were prepared as shown in Figure 1 and Supplemental Figure 2.

The microsporidian spore cells in the previous section and the yeast cells were each milled manually on an FEI Helios NanoLab 650. The spore cells were prepared using a Quorum PP3000T prep chamber and cryo-stage while the yeast cells were prepared using a Leica EM VCT500 cryo-stage. The spore cells were first trench milled orthogonally in a flat holder while the yeast cells were trench milled at a 48° angle in a tilted holder, then excess bulk material below the slabs was milled away prior to lamellae thinning.

The *E. coli* cells and HEK 293S cells were each milled on a TFS Aquilos 2 using AutoTEM to automate the lamellae thinning process after manual trench milling. First, trench milling for both cells was performed in a tilted holder orthogonally to the grid by rotating the stage, then notches were milled close to the lamellae milling angle, followed by automated overnight lamellae milling. Supplemental Video 4 shows a timelapse of AutoTEM coarse-to-fine milling.

The manually-milled yeast lamella represents a successful attempt to create the largest waffle lamella and resulted in a lamella of size 70 µm x 25 µm in length and width, and 250 – 500 nm thick (Supplemental Figure 5). Figure 4a shows a tomogram slice-through from the lamella and Supplemental Video 5 shows two representative tomograms.

The resulting AutoTEM-automated lamellae for the *E. coli* and HEK 293S cells were each about 12 µm x 12 µm in length and width, and 200 – 250 nm thick. Supplemental Video 6 shows two representative tomograms from the *E. coli* waffle lamellae. The *E. coli* cells expressed *Leviviridae* PP7 virus capsid proteins, which can be seen assembled as virus capsids inside of the bottom-right *E. coli* cell in Figure 4b and in the left tomogram in Supplemental Video 6, thus exemplifying the advantage of milling dozens of cells per lamellae using the waffle method compared to conventional cryo-FIB milling of individual or small clumps of *E. coli* cells. Figure 4c and Supplemental Video 7 shows a tomogram from the HEK 293S waffle lamellae. The cell membranes show several dozen receptors of interest, exemplifying the common need for higher throughput cryo-FIB sample preparation to study membrane-bound proteins. A low-magnification tomogram of the E. coli sample (Supplemental Video 8) shows that sections of the lamella contain complete outer membranes of top-views and side-views of the cells, illustrating further how the Waffle Method may simplify the study of outer membrane-bound proteins.

Discussion

Cryo-FIB/SEM has shown enormous promise in studying biological systems in three-dimensions at the highest resolution available. For instance, the ability to study drug delivery processes at the molecular level in native cells has the potential to change drug development, and the ability to examine disease phenotypes in patient tissue at the individual protein level may alter personalized medicine. Several bottlenecks in the cryo-FIB/SEM sample preparation portion of the conventional cryo-FIB/SEM workflow remain before these visions may potentially become routine and mainstream, such as milling specimens with preferred orientation, low-throughput due to specimen size and/or concentration, and specimen vitrification issues.

To address these issues, we present the Waffle Method for cryo-FIB/SEM specimen preparation. We illustrated the advantages of the Waffle Method by applying it to microsporidian spores to obtain missing orientations of the spores and the polar tube inside them, and to increase concentration and throughput. We illustrated the broadness of the Waffle Method by applying it to three additional specimens - yeast *S. cerevisiae*, *E. coli*, and HEK 293S cells - and to different cryo-FIB/SEM hardware. Other directionally-oriented organelles inside the cells may also be studied now with the Waffle Method. Additionally, the Waffle Method may present opportunities for cell-cell interaction studies. Several developments and implementations were key to the success of the development of the Waffle Method.

Waffle Method success required several key components and one key development. Three key components that resolve the critical issue of waffle lamella curtaining are (1) polishing planchette hats which substantially smooths the initial waffle surface, creating sufficiently smooth waffle tops (Supplemental Figure 1), (2) depositing a sufficient amount of platinum GIS prior to FIB-milling to protect the lamella during milling (Supplemental Figure 4), and (3) consistently milling lamella with the FIB beam initially intersecting the platinum GIS coat. Other key components for the Waffle Method workflow include: (4) the addition of ~25 nm carbon coat on the EM grid prior to specimen application and freezing, which strengthens the substrate to help reduce broken squares, (5) using 1-hexadecene to allow the planchette hats and optional spacer to be more easily separated after high-pressure freezing, (6) milling lamellae in parallel to reduce lamellae contamination. Lastly, (7) the development of notch milling was key for substantially increasing the likelihood of lamellae surviving preparation and grid transfers.

Notch milling in particular proved to be a critical part of Waffle Method development. Initially, gap milling similar to Wolff et al.[20] was independently developed and tested prior to any publications (Supplemental Figure 6), including milling gaps directly into the sides of lamellae, but did not appreciably increase the survivability of waffle lamellae. Due to the substantial inflexibility of the frozen waffle slab on the grid, any directional or angular forces on the grid are primarily transferred to the lamellae, which destroy them. Subsequently, a new stress-relief milling design called notch milling was developed that substantially increases the resiliency of lamellae without causing an increase in lamellae drift when collecting cryo-ET tilt-series. Notches are milled part-way through coarse lamellae milling where one side of the future lamella is completely milled through using the pattern shown in Figure 2. The notch milling pattern provides freedom of movement for the lamellae to absorb physical and thermal directional and angular forces on the grid in any direction (Supplemental Figure 7 and Supplemental Video 9). We hypothesize that notch milling provides support when the grid is not experiencing impulses by providing places for the lamellae to rest at one or more points along the length of the notch. We have observed several instances where the notch tab appears to be safely in contact with the apposed notch (e.g. Supplemental Figure 8). In our experience, this support is sufficient for lamellae survival during grid transfer and lamellae stability during cryo-ET tilt-series collection.

Currently, we routinely freeze and mill waffle grids using the Aquilos 2 and AutoTEM with high success rates. Within 1 hour, we can reliably freeze 4 waffle grids, each of which have multiple squares suitable for milling. Then, during an ~18 hour cryo-FIB/SEM session (~6 hours of trench milling and waffle lamellae site preparation, followed by ~12 hours of automated overnight milling), we can mill about 7 waffle lamellae of size 12 x 12 µm, each 200 – 250 nm thick. On the second day, we can typically collect dozens of cryo-ET tilt-series from all lamellae. Nearly 100% of waffle lamellae survive both lamellae milling and grid transfers. We find, however, that lamellae occasionally show devitrified areas. Sometimes on a single grid there will be properly vitrified lamellae and lamellae with some devitrified areas. Vitrification solutions are currently being explored.

Currently, we find that routinely milling lamellae in a semi-automated fashion using AutoTEM at a size of 12 x 12 µm is optimal for positioning lamellae windows during milling, targeting cryo-ET locations, and for routinely high lamellae survivability. We are also testing milling much larger lamellae, such as the ~70 x 25 µm lamella in Supplemental Figure 5. We

find that as lamella size increases, survivability decreases. We are currently exploring reliable solutions.

There are several optional and alternative steps in the Waffle Method:

- If there are vitrification issues due to the planchette hats not being completely full prior to high-pressure freezing, then it may be useful to apply 2-methylpentane to the nearly-assembled waffle after adding the sample. After high-pressure freezing, the 2-methylpentane may be sublimed away, leaving a properly vitrified sample as described in Harapin et al.[18]
- Less sample may be placed in the center of the grid to reduce torques and stresses on the squares and the lamellae. We hypothesize that this helps with broken lamellae because additional empty squares on the grid absorb more stress, thus reducing stress on the lamellae.
- If the sample is too viscous, then instead of pipetting sample onto the grid, sample may be smeared onto the grid like peanut butter.
- Grid spacer(s) may be used, as depicted in Figure 1 and Supplemental Video 1, to position the waffle closer to the center of the grid or to attempt to thicken the waffle. Positioning the waffle towards the center of the grid may increase the flexibility of the grid+waffle as hypothesized in the point above, which may in turn increase the survivability rate of waffled lamellae. Thickening the waffle may be useful for particularly large specimens, however caution should be taken not to make the waffle thicker than about 50 µm, which is the typical depth of focus for cryo-FIB milling during fine milling[11]. The ~50 µm thickness limit may be circumvented by first preparing a thicker waffle, trench milling wide trenches at a moderate angle, then milling the bottom off at shallower angles until the remaining slab is less than 50 µm thick, and finishing milling. This strategy will only leave the top ~50 µm of the slab available for milling, which may limit the tilt angle range during cryo-ET collection if the trenches are not large enough.
- Planchette hats of different sizes may be used to optimize waffle thickness.
- It may be useful to add a grid to the top of the sample (ie. between the sample and the top planchette in Figure 1f) during waffle assembly to try to force it to be flat on top.
- The waffle may be assembled outside of the HPF tip. We had the best success preparing it inside the HPF tip as shown in Supplemental Figure 2 and Supplemental Video 2.

- Platinum GIS may be applied to the side of freshly-cut trenches to allow for milling lamellae with the leading edge on the side of the trench rather than the top.
- An arrowhead notch (Supplemental Figure 9) may be used instead of the proposed notch in this manuscript. We have not tested if there is a difference in lamellae survivability depending on which notch type is used.
- A cryo-FLM may be used to optimize localization of objects of interest at any stage during specimen preparation on the grid.
- A magnifying glass and/or a camera may be used when positioning the grid into the cryo-FIB/SEM holder and the cryo-EM holder for cryo-ET collection to orient the squares to be parallel or perpendicular to the tilt range of the beam as necessary.

Additionally, we have explored using 50 mesh and 100 mesh grids in order to increase the size of the lamellae. However, in our experience the grid and lamellae become substantially less stable due to the larger squares.

There are several applications of the Waffle Method that are currently being explored. Figure 5 depicts three potential applications of the Waffle Method. (1) Long complexes like filaments and microtubules may be studied from all directions (Figure 5a), as opposed to in conventional single particle preparation where axial views do not exist. (2) Single particle proteins may be studied in bulk solution after waffle milling, which will circumvent any issues caused by the air-water interface or substrate-protein interface in conventional single particle preparation, including denaturation, aggregation, preferred orientation, no particles in holes, etc.[15–17] (Figure 5b). (3) Long, skinny cells, such as the *Spirochaeta plicatilis* depicted in Figure 5c, may be studied in all orientations. Each of these potential applications will inherently result in partially milled objects that will need to be removed or avoided during processing.

A preliminary experiment suggests that applying the Waffle Method to single particle specimens is possible. Figure 4d and Supplemental Video 10 shows a tomogram of porcine thyroglobulin (ThG) as a single particle sample from a waffle lamella. The ThG sample, prepared at 100 mg/mL, shows randomly oriented ThG particles that are mono-dispersed in 3D. Based on the bulk sample concentration and the number of observed particles in the tomograms, we estimate that the density of particles in bulk and in the lamellae are roughly the same, suggesting that the Waffle Method and recent cryo-ET processing algorithms[21] may solve all conventional single particle cryo-EM air-water interface issues. However, further studies in this direction across several specimens are required before definitive conclusions may be made.

There are several other issues that the Waffle Method may alleviate. (1) The Waffle Method may be used to study cells that do not behave on conventionally-prepared EM grids due to suboptimal grid affinity, suboptimal concentration in grid squares, wicking/hydration issues, etc. Suboptimal grid affinity may occur because some cells prefer to adhere to grid bars, for example. Suboptimal concentration in grid squares may be defined as sample concentration differences between the time before applying the sample to the grid and after freezing. Wicking/hydration issues on grids include cells that are so dry that the biological significance is impacted and grids that are so wet that the cells are difficult to locate after freezing or that are not properly vitrified. (2) Due to the random location and orientation of cells in waffles lamellae, there will be cross-sections of outer cell membranes in waffles (Supplemental Video 8), which are difficult or impossible to obtain with conventional cryo-FIB/SEM. (3) Tissues and organisms may be studied using the Waffle Method instead of performing cryo-FIB/SEM liftout.

Compared to cryo-liftout, the Waffle Method may have several benefits. (1) Less specialized hardware is required; liftout requires an in-chamber tool, separate grid holder, and different grids, while the Waffle Method only requires a high-pressure freezer. (2) Less operator specialization is required. (3) Higher-throughput may be achieved; more lamellae can be made per grid. (4) Finally, the Waffle Method is more automatable due to all cryo-FIB/SEM operations being performed on one grid.

Here, we presented three waffled specimens that took advantage of the Aquilos 2 AutoTEM lamellae milling automation software. Specifically, the steps that we automated were coarse-to-fine milling and polishing (Supplemental Video 4). Currently, waffle lamellae locations must first be manually trench milled and manually notch milled before automated lamellae coarse-to-fine milling can be performed in AutoTEM due to the current limits of the software. We anticipate that all waffle milling steps will be automated in the future.

The Waffle Method may not provide all of its benefits to adherent cells that stop replicating after confluence and do not form multiple layers. However, these types of waffled adherent cells may benefit from proper vitrification due to high-pressure freezing and from the large lamellae that waffle milling produces (conventional cryo-FIB/SEM cannot provide as large of lamellae even with clumped cells because the top-surface of the cells is not topologically uniform). However, the lamellae will be mostly empty except for the bottom of the lamellae that will contain slices of cells as desired. Shorter planchette hats may be used to optimize waffle thickness, and possibly development of grids with shorter grid bars. As a result, throughput may be slightly increased compared to conventional cryo-FIB/SEM.

We anticipate that the Waffle Method will broaden the specimens amenable to cryo-FIB/SEM while solving preferred orientation issues, increasing throughput during both cryo-FIB/SEM and cryo-ET, allowing small and skinny cells to be studied, allowing cells that behave poorly on grids to be studied, and providing good vitrification for specimen of all sizes. We also envision that the Waffle Method may be used as a platform for further method development in cryo-FIB/SEM and cryo-EM. The Waffle Method may also become a way of performing cryo-liftout on the grid, particularly for tissue specimens. Additionally, we anticipate that the Waffle Method will be fully automated to increase throughput and reduce operator time.

Methods

Several variations of the Waffle Method for the different specimens and hardware in this manuscript are described here. We note that implementation of the Waffle Method with new specimens and hardware may require a new combination of these methods.

Microsporidian spore sample preparation

The *A. algerae* microsporidian spores shown in Figure 3b were propagated in *Helicoverpa zea* larvae and purified using a continuous Ludox gradient, as previously described[22]. *E. hellem* microsporidian spores (ATCC 50504) were used in Figure 3a,c-g. The spores were propagated in Vero cells (ATCC CCL-81). Vero cells were maintained in Eagle's Minimum Essential Medium (EMEM) (ATCC 30–2003) with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C with 5% CO_2. At ~90% confluence, the media was replaced with EMEM supplemented with 3% FBS and the parasites were added into a 25 cm^2 tissue culture flask. Medium was changed every 2 days. After 14 days post-infection, the cells were detached from the flask using a cell scraper and centrifuged at 1,300 g for 10 minutes at room temperature. Cell pellets were resuspended with 5 mL of distilled water and mechanically disrupted using a G27 needle. To purify microsporidian spores, 5 mL of a 100% Percoll was added and vortexed prior to centrifugation at 1,800 g for 30 minutes at 25 °C. The purified spore pellets were washed 3 times with 1X PBS and stored at 4 °C until use. Spore concentration (1.54×10^8 spores/mL) was measured using a hemocytometer.

Microsporidian spore waffle grid preparation

The general Waffle Method workflow is described in the Results section. Here and in the other waffle preparation sections for the other specimen, we describe the specific equipment and workflows used during waffle milling. This section on microsporidian spore waffle grid preparation covers the period of Kotaro's Waffle Method development, and is accurate to the best of our knowledge. Waffle Method development resulted in the following protocol.

We use Quantifoil carbon 200 mesh EM grids (Quantifoil, Jena, Germany). About 25 nm of carbon is sputtered onto the non-grid bar side of the grid using a Leica EM ACE600 High Vacuum Sputter Coater (Leica Biosystems, Germany). Planchette hats (3.3 mm diameter) are polished for several minutes using POL Metallpflege metal-polish and a Kimwipe. Excess is

wiped off using a Kimwipe. The planchette hats and grid spacer are coated with 1-hexadecene. The grid is plasma cleaned using a Gatan Solarus (Gatan Inc, Pleasanton, CA) with the following recipe: 80% O_2 gas and 20% H_2 gas for 30 seconds.

The HPF tip is cleaned with ethanol prior to assembling the bottom of the waffle in the HPF tip as shown in Supplemental Video 2. Several microliters of sample (typically 3 – 6 µL) are applied to the bottom waffle assembly on the grid bar side such that no air pockets are present, and optionally 2-methylpentane is applied to the top of the sample just prior to placing the top planchette hat on and closing the HPF tip. The assembled waffle grid in the HPF tip is quickly transferred to a Wohlwend HPF Compact 01 high-pressure freezer where it is high-pressure frozen. The HPF tip is then disassembled to release the waffled grid inside of the planchette hats. The planchette hats are disassembled using a combination of tweezers, flat screwdrivers, and razor blades to reveal the waffled grid. The waffle grid is then carefully clipped.

The grid is placed in a flat or tilted cryo-FIB/SEM holder and inserted into the Quorum PP3000T prep chamber (Quorum Technologies, Great Britain) attached to the FEI Helios NanoLab 650 cryo-FIB/SEM (FEI, Hillsboro, OR) at NYSBC. Conductive platinum is sputtered onto the waffled grid inside of the prep chamber for 60 seconds before placing the shuttle onto the cold stage in the main chamber. Platinum GIS is deposited onto the waffle for 4 – 7 seconds at 35 °C at 7 mm from working distance. Grid bar lines are drawn into the waffle to help locate areas of interest and orient the cryo-FIB/SEM operator. Two trenches tens of microns in dimensions and about 30 µm apart for each area of interest are milled at between 45° and 90° from the grid plane with a milling current between 9.3 nA and 0.79 nA. If trench milling is performed far from 90°, the tilt angle is decreased several times by 5° while milling the bottom area under the slab to ensure that no material remains. The grid is placed into a tilted holder, if not already in one, and placed back into the cryo-FIB/SEM chamber. Eucentric height is obtained for each trenched slab location and positions are saved at shallow angles below 20° from the grid plane. All subsequent milling is performed in parallel* across all lamellae and with the FIB beam first intersecting the platinum GIS layer** to minimize curtaining. The slabs are coarse milled with a milling current between 2.5 nA and 0.43 nA until the slab is about 3 µm thick. A notch is milled into one side of the slab as described in the main text and as shown in Figure 2. Current is reduced to 0.23 nA and the slab is milled down to 1.5 µm thick with a tab left on the lamella beside the notch while tilting the stage ±1° and while milling more on the carbon side than the sample side. Current is reduced to 80 pA (using the cleaning cross section (CCS) pattern type) and the lamella is milled down to 0.5 µm thick while tilting the stage ±0.5°. With

current still at 80 pA CCS and without tilting, the lamella is milled down to the desired final thickness (usually 200 nm or less) before polishing. The grid is removed from the prep chamber and conductive platinum is sputtered onto the grid and lamellae for several tens of seconds.
*Milling in parallel means to first magnify to the location of slab 1 for trench milling, slab 2 for trench milling, slab 3 …, then slab 1 for coarse milling, slab 2 for coarse milling, slab 3 …, then lamella 1 for fine milling, lamella 2 for fine milling, lamella 3 …, and finally lamella 1 for polishing, lamella 2 for polishing, lamella 3 …
**After trench milling, two surfaces are exposed for coarse and fine milling: (1) The frozen sample and (2) the platinum above the sample. All coarse and fine milling should first hit (2) the platinum above the sample. This will minimize curtaining.

Microsporidian spore Cryo-ET collection

Tilt-series were collected with Leginon[23] using a TFS Titan Krios (Thermo Fisher Scientific) using counting mode on a Gatan K2 BioQuantum with the energy filter slit width set to 20 eV (NYSBC Krios #3). Tilt-series were collected with a nominal defocus of -6 µm, pixelsize of 3.298 Å, 1.13 – 1.74 e-/Å^2 dose per tilt image where dose was increased with the cosine of the tilt angle resulting in 15 – 23 frames per tilt image and 68 e-/Å^2 total dose per tilt-series. Collection was performed bi-directionally from [0:50]° then [0:-50]° with 2° tilt increments. A total of 27 tilt-series were acquired.

Microsporidian spore Cryo-ET processing

Tilt images were frame aligned with MotionCor2[24] without patches or dose weighting. Frame aligned tilt images were used for fiducial-less tilt-series alignment in Appion-Protomo[25–27]. Only tilt images from [-50:20]° were used for the tomogram in Figure 3e due to excessive stage drift. All tilt images were used for the right tomogram in Figure 3f. Tilt-series were dose weighted in Appion-Protomo using equation 3 in Grant & Grigorieff[28] prior to reconstruction with Tomo3D[29,30] SIRT, then denoised using the Topaz-Denoise[31] pre-trained model. Tomograms were visualized with IMOD[32].

Conventional cryo-FIB/SEM grid preparation

3 µL of *Anncaliia algerae* spores (6.8 × 10^7 spores/mL) was applied to a Quantifoil holey carbon grid on copper support (2/2, 400 mesh) glow discharged for 1 minute, and back-blotted for 15

seconds. Then, the sample was frozen in liquid ethane using the Leica EM GP plunge freezer. The grid was screened on an FEI Talos Arctica cryo-TEM at the cryo-EM Shared Resource at NYU School of Medicine. The FEI Helios NanoLab 650 at NYSBC was used to perform conventional cryo-FIB/SEM. Briefly, the sample was platinum coated in the Quorum stage, a couple microns of platinum GIS was applied in the Helios main chamber, and spores were individually milled at shallow angles (< 15°).

Yeast cells sample preparation

The yeast strain *Saccharomyces cerevisiae* YOL058W was grown on solid YPD media at 30 °C. Cells were harvested from the plate and centrifuged in liquid YPD media for 4 minutes at 3,000 rpm at 4 °C. The cell pellet was resuspended just prior to grid preparation.

Yeast cells waffle grid preparation

The waffle grid was prepared in the same manner as the 'Microsporidian spore waffle grid preparation' section, except for the following differences. Planchette hats were sanded down using 1,200, 7,000, then 15,000 grit sandpaper until smooth. POL Metallpflege metal-polish was applied to the smooth side of the hats and polished on filter paper, then any excess polish was wiped off using a Kimwipe. Immediately before assembling, both planchette hats were rinsed with 1-hexadecene. The waffle grid was assembled in the HPF tip as follows: planchette hat (smooth side up), glow-discharged copper Quantifoil grid with 25 nm of extra carbon on the film layer (copper grid bar side up), 5 µL of resuspended cell pellet, planchette hat (smooth side down). Excess sample was wiped off with Kimwipes. This assembly was then quickly inserted into a Wohlwend HPF Compact 01 and high-pressure frozen. The waffle was then disassembled under liquid nitrogen and clipped into an autogrid.

Yeast cells waffle milling

The waffle grid was placed into a 40° pre-tilted cryo-FIB/SEM grid holder (Leica Microsystems) and inserted into the FEI Helios NanoLab 650 FIB/SEM (Thermo Fisher Scientific) with Leica EM VCT500 cryo-stage at NYSBC. A grid overview image was taken and centrally-located squares with an even surface were selected. The grid was then coated with platinum GIS for 1 minute. The grid was then rotated 180° (175° to -5°) and tilted to 60° to mill at a 48° angle relative to the ion beam. Two rectangle trenches were milled at 9.3 nA with a spacing of 15 µm.

The grid was then rotated back to the original position and tilted to a milling angle of 5°. Any visible bulk material was removed at 2.5 nA prior to thinning. Each lamella was milled manually following the parameters in Table 2. The first step of the relief notch was milled at 0.23 nA when the lamella was 2 µm in thickness and the final step at 1 µm. Another clean-up of excess material was performed at 2.5 nA at 25° stage tilt prior to the final polishing steps.

	Lamella thickness (µm)	Milling angle	Milling Current (nA)	Pattern Type
Rough Milling	3	17°	0.79	Rectangle
Medium Milling	2	17°	0.43	Rectangle
Fine Milling	1.5	17°	0.23	Rectangle
Over/Under-tilt	1	16.5°, 17.5°	0.23	CCS
Polishing 1	0.30	17°	0.080	CCS
Polishing 2	0.20	17°	0.024	CCS

Table 2: Manual FIB-milling and thinning parameters.

Yeast cells cryo-ET collection

Tilt-series were collected with Leginon[23] using a TFS Titan Krios (Thermo Fisher Scientific) with a Cs corrector and using counting mode on a Gatan K3 BioQuantum with the energy filter slit width set to 20 eV (NYSBC Krios #2). Tilt-series were collected with a nominal defocus of -4 µm, pixelsize of 2.077 Å, 4.15 – 6.23 e-/Å^2 dose per tilt image where dose was increased with the cosine of the tilt angle resulting in 12 – 18 frames per tilt image and 180 e-/Å^2 total dose per tilt-series. Collection was performed bi-directionally from [0:48.6]° then [0:-48.6]° with 2.7° tilt increments. A total of 30 tilt-series were acquired.

Yeast cells cryo-ET processing

Tilt images were frame aligned with MotionCor2[24] without patches or dose weighting. Frame aligned tilt images were used for fiducial-less tilt-series alignment in Appion-Protomo[25–27]. Tilt-series were dose weighted in Appion-Protomo using equation 3 in Grant & Grigorieff[28] prior to reconstruction with Tomo3D[29,30] SIRT, then denoised using the Topaz-Denoise[31] pre-trained model. Tomograms were visualized with IMOD[32].

E. coli sample preparation

E. coli BL21 (DE3) cells were harvested by centrifugation at 6,000 g four hours post-induction of *Leviviridae* PP7-PP7 protein production as previously described[33]. Resulting cell pellet was resuspended in equal volume of phosphate-buffered saline buffer (PBS) and kept at 4 °C for ~48 hours during shipment from Georgia Tech to NYSBC.

E. coli waffle grid preparation

The waffle grid was prepared in the same manner as the 'Microsporidian spore waffle grid preparation' section, except for the following differences. Planchette hats (3.3 mm diameter) are polished using following workflow: (1) Rub with coarse sandpaper (grit 1,200) to remove machining marks from the hats, (2) Rub with fine sand paper (grit 15,000), and (3) Final polish for 10 seconds with POL Metallpflege metal-polish and a Kimwipe (Supplemental Figure 1). The waffle grid was assembled in the HPF tip as the following: planchette hat (smooth side up), prepared Quantifoil 2/2 200 mesh Cu grid with 25 nm extra carbon on the film layer (copper grid bar side up), 5 µL of induced *E. coli* BL21 (DE3) sample, planchette hat (smooth side down). Any excess sample that spilled out of the assembly was wiped off with a Kimwipe. This assembly was then quickly inserted into a Wohlwend HPF Compact 01 and high-pressure frozen. The waffle was then disassembled under liquid nitrogen and clipped into a notched autogrid.

E. coli waffle milling

The waffle grid was placed into a 45° pre-tilted cryo-FIB/SEM shuttle and inserted into the Aquilos 2 cryo-FIB/SEM (Thermo Fisher Scientific) at NYSBC. A mosaic of the grid was taken in TFS MAPS 3.16, and squares with an even surface were visually identified. A conductive platinum layer was then sputtered on the grid for 15 seconds at 30 mA and 10 Pa. The grid was then put in deposition position and coated with platinum GIS for 2 minutes. The grid was then rotated 180° (-71.9° to 108.1°) and tilted to 7° to make the grid surface orthogonal to the ion beam. Two trenches were milled at 15 nA with a spacing of 25 µm. The top trench (closer to the grid notch) was 22 µm wide and 37 µm long, while the bottom trench (further from the grid notch) was 20 µm wide and 17 µm long. The grid was then rotated back to the original position and tilted to a milling angle of 26°. The trench closest to the notch was then milled again at this angle to clean up bulk material below the lamellae.

Using AutoTEM Cryo 2.2, the preparation step, which includes eucentric tilting, setting the milling angle, collecting a reference image, and lamella placement, is done for each lamella site. Another cleanup of the trench closer to the grid notch, at 3 nA, and a relief notch, at 0.1 nA, is milled at an angle of 20°. Each lamella placement is then repositioned about 1 µm from the relief notch. Each lamella is then milled and thinned stepwise using the parameters in Table 1.

	Lamella thickness (defined as "Pattern Offset") (µm)	Front Width Overlap (µm)	Rear Width Overlap (µm)	Milling Current (nA)	Pattern Type	DCM Rescan Interval (s)
Rough Milling	1.0	1.5	1.0	1.0	Rectangle	120
Medium Milling	0.8	0.65	0.5	0.5	CCS	90
Fine Milling	0.6	0.35	0.1	0.3	CCS	60
Finer Milling	0.4	0.05	0.05	0.3	CCS	30
Polishing 1	0.15	N/A	N/A	0.03	CCS	30
Polishing 2	0	N/A	N/A	0.01	CCS	10

Table 1: AutoTEM Cryo 2.2 FIB-milling and thinning parameters. Milling and thinning were done at angle 20°, and a depth correction of 100% and 0° overtilt at 30 kV while polishing steps were set to 160%.

E. coli cryo-ET collection

Tilt-series were collected with Leginon[23] using a TFS Titan Krios (Thermo Fisher Scientific) with a Cs corrector and using counting mode on a Gatan K3 BioQuantum with the energy filter slit width set to 20 eV (NYSBC Krios #2). Tilt-series were collected with a nominal defocus of -4 to -8 µm, pixelsize of 2.077 Å, 4.87 – 9.39 e-/Å^2 dose per tilt image where dose was increased with the cosine of the tilt angle resulting in 14 – 27 frames per tilt image and 234 e-/Å^2 total dose per tilt-series. Collection was performed bi-directionally from [0:48]° then [0:-58]° with 2.8° tilt increments. A total of 41 tilt-series were acquired.

E. coli cryo-ET processing

Tilt images were frame aligned with MotionCor2[24] without patches or dose weighting. Frame aligned tilt images were used for fiducial-less tilt-series alignment in Appion-Protomo[25–27]. Tilt-series were dose weighted in Appion-Protomo using equation 3 in Grant & Grigorieff[28] prior to reconstruction with Tomo3D[29,30] SIRT, then denoised using the Topaz-Denoise[31] pre-trained model. Tomograms were visualized with IMOD[32].

HEK 293S cells sample preparation

Suspension HEK 293S GnTI$^-$ cells (ATCC) were cultured in the Freestyle 293 expression medium (GIBCO) at 37 °C and 5% CO_2. A 10 mL aliquot was centrifuged for 5 minutes at 500 g at room temperature, then the resulting cell pellet was resuspended to a final concentration of 3.5e8 in culturing media.

HEK 293S cells waffle grid preparation

The waffle grid was prepared in the same manner as the 'Microsporidian spore waffle grid preparation' section, except for the following differences. Planchette hats were sanded down using 1,200, 7,000, then 15,000 grit sandpaper until smooth. POL Metallpflege metal-polish was applied to the smooth side of the hats and polished on filter paper, then any excess polish was wiped off using a Kimwipe. Immediately before assembling, both planchette hats were rinsed with 1-hexadecene. The waffle grid was assembled in the HPF tip as follows: planchette hat (smooth side up), glow-discharged gold Quantifoil grid with 20 nm of extra carbon on the film layer (copper or gold grid bar side up), 4 µL of resuspended cell pellet, planchette hat (smooth side down). Excess sample was wiped off with blotting paper #4. This assembly was then quickly inserted into a Wohlwend HPF Compact 01 and high-pressure frozen. The waffle was then disassembled under liquid nitrogen and clipped into a notched autogrid.

HEK 293S cells waffle milling

The waffle grid was placed into a 45° pre-tilted cryo-FIB/SEM shuttle and inserted into the Aquilos 2 cryo-FIB/SEM (Thermo Fisher Scientific) at NYSBC. A grid overview was taken in MAPS 3.16, and squares subjectable for milling were targeted as Lamella sites. A conductive platinum layer was then sputtered on the grid for 15 seconds at 30 mA and 10 Pa. The grid was

then put in deposition position and coated with platinum GIS for 2 minutes. The grid was then rotated 180° (actual angles -71.9° to 108.1°), tilted to 7° to make the grid surface orthogonal to the ion beam. Two trenches were milled at 15 nA with a spacing of 25-30 µm. The top trench (closer to the grid notch) was 22 µm wide and 32-37 µm long, while the bottom trench (further from the grid notch) was 20 µm wide and 17 µm long. The grid was then rotated back to the original position. Using AutoTEM Cryo 2.2, the “Preparation” step is done in guided mode for each lamella site, which includes eucentric tilting, setting the milling angle (was set to 20°), collecting a reference image, and an initial lamella placement. Then, bulk material below the lamellae were cleaned up at 7 nA (at maximum stage tilt angle) and 3 nA (at milling angle) until there was no visible leftover material left. After the cleanup, a notch was added at the milling angle at 0.3 nA. The total length of the notch was about 5 um. Ultimately, the image acquisition and lamella placement steps in “Preparation” were repeated for each site while repositioning the lamella about 1 µm away from the milled notch. Each lamella is then milled and thinned with AutoTEM in stepwise mode using the parameters from Table 1.

HEK 293S cells cryo-ET collection

Tilt-series were collected with Leginon[23] using a TFS Titan Krios (Thermo Fisher Scientific) with a Cs corrector and using counting mode on a Gatan K3 BioQuantum with the energy filter slit width set to 20 eV (NYSBC Krios #2). Tilt-series were collected with a nominal defocus of -4 to -5 µm, pixelsize of 1.632 Å, 4.12 – 5.83 e-/Å^2 dose per tilt image where dose was increased with the cosine of the tilt angle resulting in 12 – 17 frames per tilt image and 180 e-/Å^2 total dose per tilt-series. Collection was performed bi-directionally from [0:59]° then [0:-39]° with 2.8° tilt increments. A total of 6 tilt-series were acquired.

HEK 293S cells cryo-ET processing

Tilt images were frame aligned with MotionCor2[24] without patches or dose weighting. Frame aligned tilt images were used for fiducial-less tilt-series alignment in Appion-Protomo[25–27]. Tilt-series were dose weighted in Appion-Protomo using equation 3 in Grant & Grigorieff[28] prior to reconstruction with Tomo3D[29,30] SIRT, then denoised using the Topaz-Denoise[31] pre-trained model. Tomograms were visualized with IMOD[32].

ThG sample preparation

Porcine thyroglobulin from porcine thyroid gland (Sigma T1126) was dissolved in 50 mM HEPES pH 7.4 and 100 mM NaCl. The final concentration was about 100 mg/ml.

ThG waffle grid preparation

The waffle grid was prepared in the same manner as the 'Microsporidian spore waffle grid preparation' section, except for the following differences. Planchette hats (3.3 mm diameter) are polished using following workflow: (1) Rub with coarse sandpaper (grit 1,200) to remove machining marks from the hats, (2) Rub with fine sand paper (grit 15,000), and (3) Final polish for 10 seconds with POL Metallpflege metal-polish and a Kimwipe (Supplemental Figure 1). The waffle grid was assembled in the HPF tip as the following: planchette hat (smooth side up), prepared Quantifoil 2/2 200 mesh Cu grid with 25 nm extra carbon on the film layer (copper grid bar side up), 5 µL of 100 mg/ml porcine Thyroglobulin sample, planchette hat (smooth side down). Any excess sample that spilled out of the assembly was wiped off with a Kimwipe. This assembly was then quickly inserted into a Wohlwend HPF Compact 01 and high-pressure frozen. The waffle was then disassembled under liquid nitrogen and clipped into a notched autogrid.

ThG waffle milling

The waffle grid was placed into a 45° pre-tilted cryo-FIB/SEM shuttle and inserted into the Aquilos 2 cryo-FIB/SEM (Thermo Fisher Scientific) at NYSBC. A mosaic of the grid was taken in TFS MAPS 3.16, and squares with an even surface were visually identified. A conductive platinum layer was then sputtered on the grid for 15 seconds at 30 mA and 10 Pa. The grid was then put in deposition position and coated with platinum GIS for 2 minutes. The grid was then rotated 180° (-71.9° to 108.1°) and tilted to 7° to make the grid surface orthogonal to the ion beam. Two trenches were milled at 15 nA with a spacing of 25 µm. The top trench (closer to the grid notch) was 22 µm wide and 37 µm long, while the bottom trench (further from the grid notch) was 20 µm wide and 17 µm long. The grid was then rotated back to the original position and tilted to a milling angle of 26°. The trench closest to the notch was then milled again at this angle to clean up bulk material below the lamellae.

Using AutoTEM Cryo 2.2, the preparation step, which includes eucentric tilting, setting the milling angle, collecting a reference image, and lamella placement, is done for each lamella

site. Bulk material below the lamella was cleaned at 7 nA (at maximum stage tilt angle) then at 3 nA (at a milling angle of 20°). After, a relief notch is added, at 0.3 nA (at a milling angle of 20°). Each lamella placement is then repositioned about 1 µm from the relief notch. Each lamella is then milled and thinned stepwise using the parameters in Table 1.

ThG cryo-ET collection

Tilt-series were collected with Leginon[23] using a TFS Titan Krios (Thermo Fisher Scientific) with a Cs corrector and using counting mode on a Gatan K3 BioQuantum with the energy filter slit width set to 20 eV (NYSBC Krios #2). Tilt-series were collected with a nominal defocus of -5 µm, pixelsize of 1.632 Å, 3.34 – 6.67 e-/Å^2 dose per tilt image where dose was increased with the cosine of the tilt angle resulting in 9 – 18 frames per tilt image and 148 e-/Å^2 total dose per tilt-series. Collection was performed bi-directionally from [0:45]° then [0:-60]° with 3° tilt increments. A total of 15 tilt-series were acquired.

ThG cryo-ET processing

Tilt images were frame aligned with MotionCor2[24] without patches or dose weighting. Frame aligned tilt images were used for fiducial-less tilt-series alignment in Appion-Protomo[25–27]. Tilt-series were dose weighted in Appion-Protomo using equation 3 in Grant & Grigorieff[28] prior to reconstruction with Tomo3D[29,30] SIRT, then denoised using the Topaz-Denoise[31] pre-trained model. Tomograms were visualized with IMOD[32].

Acknowledgements

This manuscript is dedicated to Kotaro Kelley and his family, and serves as a memory of him. Kotaro conceived of and developed the Waffle Method, but passed away before this manuscript was written. As Kotaro was the only Waffle Method expert during microsporidia waffle preparation, we present here as much knowledge and data for these portions of the manuscript as we have access to and that we understand. Thus, this manuscript in part serves as a method to disseminate the knowledge and skills developed by Kotaro before he passed. The Waffle Method examples in Figure 3 and Supplemental Figures 3b-f, 4, & 6 were all performed by Kotaro. We are continuing to develop the Waffle Method and extensions of it as described in the Discussion. We hope Kotaro's knowledge and spirit continue to make positive impacts on people's lives through biomedical research that extend beyond and are derivatives of his research.

We thank James J. Becnel and Neil Sanscrainte for the microsporidian *A. algerae* sample and Nicolas Coudray for helping screen the grid at the cryo-EM Shared Resource at NYU School of Medicine. We thank M.G. Finn and Liangjun Zhao from Georgia Tech for providing *E.coli* BL21 cells expressing PP7-PP7 virus-like particles. We thank Sagar Khavnekar at Max Planck Institute of Biochemistry (MPIB) and Dr. Philipp Erdmann at MPIB (now at Human Technopole) for sharing their experiences with implementing the Waffle Method. We thank Viacheslav Serbynovskyi for testing potential alternative Waffle Method freezing techniques. We thank Alexander I. Sobolevsky and Maria V. Yelshanky for providing the HEK 293S cells. A.J.N. was supported by a grant from the NIH National Institute of General Medical Sciences (NIGMS) (F32GM128303). P.J. was supported by American Heart Association (19POST34430065). G.B. was supported by Pew Biomedical Scholars (PEW-00033055), Searle Scholars Program (SSP-2018-2737) and the National Institute of Allergy and Infectious Diseases (R01AI147131). Some of this work was performed at the Simons Electron Microscopy Center located at the New York Structural Biology Center, supported by grants from the Simons Foundation (SF349247), NIH NIGMS (GM103310) and NIH (U24GM139171).

Author contributions

K.K. conceived of this project, developed and implemented the Waffle Method, and prepared and collected the microsporidia waffle cryo-FIB/SEM-ET data. K.K. independently conceived and tested gap milling methods and notch milling. K.K. and A.J.N. processed and analyzed the microsporidia waffle data. A.J.N. prepared, collected, and analyzed the conventional cryo-FIB/SEM data. P.J. prepared the microsporidian spore samples. A.M.R., O.K., D.B., and M.K. prepared, waffled, and collected the yeast, *E. coli*, and HEK 293S cells. A.J.N. processed and analyzed the tomograms. K.K., A.M.R., O.K., P.J., D.B., M.K., E.E., G.B., C.S.P., B.C., and A.J.N. designed the experiments. A.J.N. wrote the manuscript, created the figures, and created the videos and animations. A.M.R., O.K., P.J., D.B., M.K., E.E., G.B., C.S.P., B.C., and A.J.N. edited the manuscript.

Data availability

Tomograms have been deposited to EMD-XXXX. Raw data has been deposited to EMPIAR-YYYYY.

Competing interests

The authors declare no competing interests.

Figure 1: General Waffle Method workflow

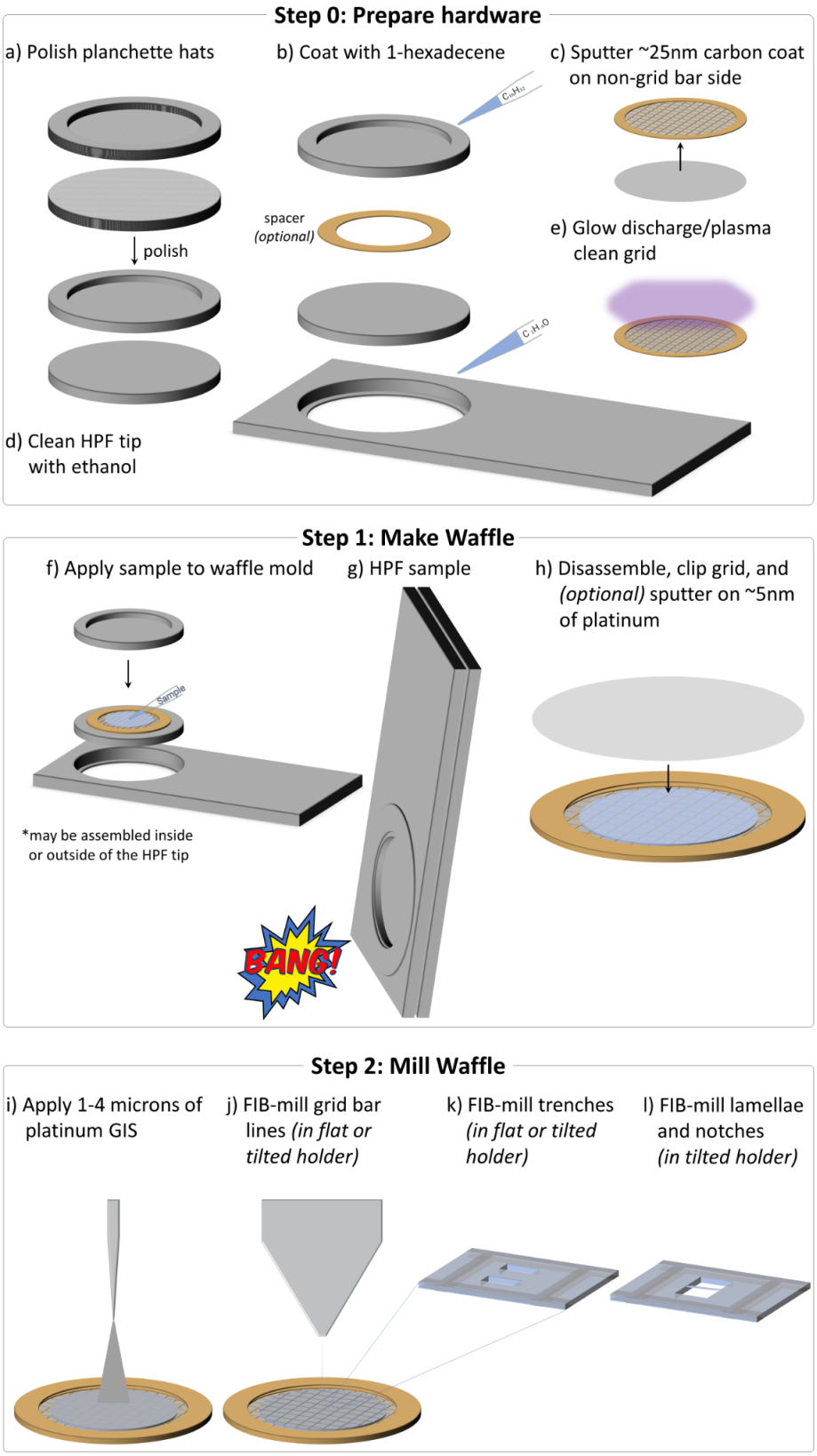

Figure 1 | Schematic of the general workflow for creating a waffle grid. Supplemental Video 1 shows an animation of all steps. In Step 0, the waffle is prepared: **(a)** The planchette hats are polished to reduce lamellae curtaining, **(b)** The planchette hats and optional spacer are coated with 1-hexadecene to make easier to separate after high-pressure freezing, **(c)** ~25 nm of carbon is sputtered onto the non-grid bar side of the EM grid to rigidify it, **(d)** The HPF tip is cleaned with ethanol just prior to use, and **(e)** the EM grid is glow discharged/plasma cleaned just prior to sample application. In Step 1, the waffle is made: **(f)** The sample is applied to the EM grid with optional spacer on top of a planchette hat, **(g)** The waffle is vitrified with the HPF, **(h)** The HPF assembly is taken apart, the grid is carefully clipped, and ~5 nm of conductive platinum is sputtered onto the waffle. The researcher may want to screen their waffle grid in a cryo-FLM at this step. In Step 2, the waffle is milled inside of a cryo-FIB/SEM: **(i)** 1 – 4 µm of platinum GIS is applied, **(j)** Grid bar lines are drawn onto the top of the waffled sample to expedite location identification during milling, **(k)** Two trenches per lamella are milled as perpendicular to the grid as possible and separated by tens of microns, **(l)** The grid is tilted to a shallow angle and all lamellae on the grid are milled in parallel while making sure the leading edge of the FIB beam always intersects platinum GIS first to reduce curtaining. It is recommended that a notch be milled into one side of each lamellae during the course of milling, as shown in Figure 2. It is recommended to tilt the stage ±1° while milling the lamellae from 3 µm thick to 1.5 µm thick and ±0.5° while milling the lamellae from 1.5 µm thick to 0.5 µm thick prior to fine milling and polishing at the desired angle. Supplemental Figure 3 shows waffle milling FIB/SEM images. Supplemental Video 2 shows most of Step 1 being performed.

Figure 2: Notch milling for waffle lamellae stress-relief

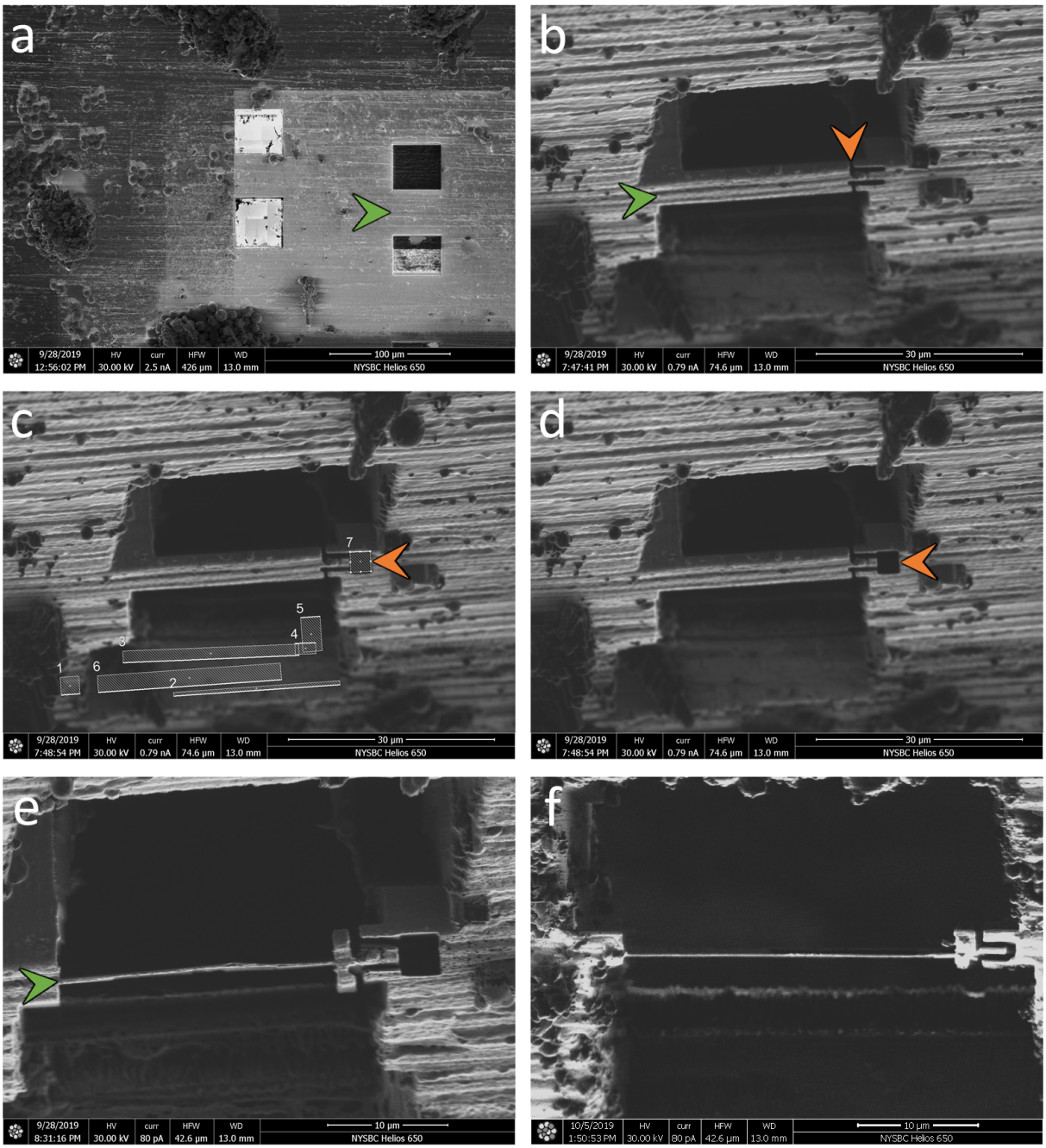

Figure 2 | FIB/SEM images of the notch milling workflow. After trench-milling **(a)** (green arrow), the resulting slab is coarsely milled down to <10 µm thick **(b)** (green arrow). The initial, incomplete notch design is milled into one side of the slab, with a segment still connected **(b)** (orange arrow). This connection is then broken by milling, revealing a tab within a notch **(c,d)** (orange arrow). The lamella is then milled and polished as usual **(e)** (green arrow). A second example of a completed lamella with a notch mill is presented in **(f)**. To allow for freedom of movement (see Supplemental Figure 7 and Supplemental Video 9), each notch mill should separate the tab from the slab by several hundred nanometers.

Figure 3: Example of the Waffle Method applied to small cells to solve preferred orientation, throughput, and concentration issues

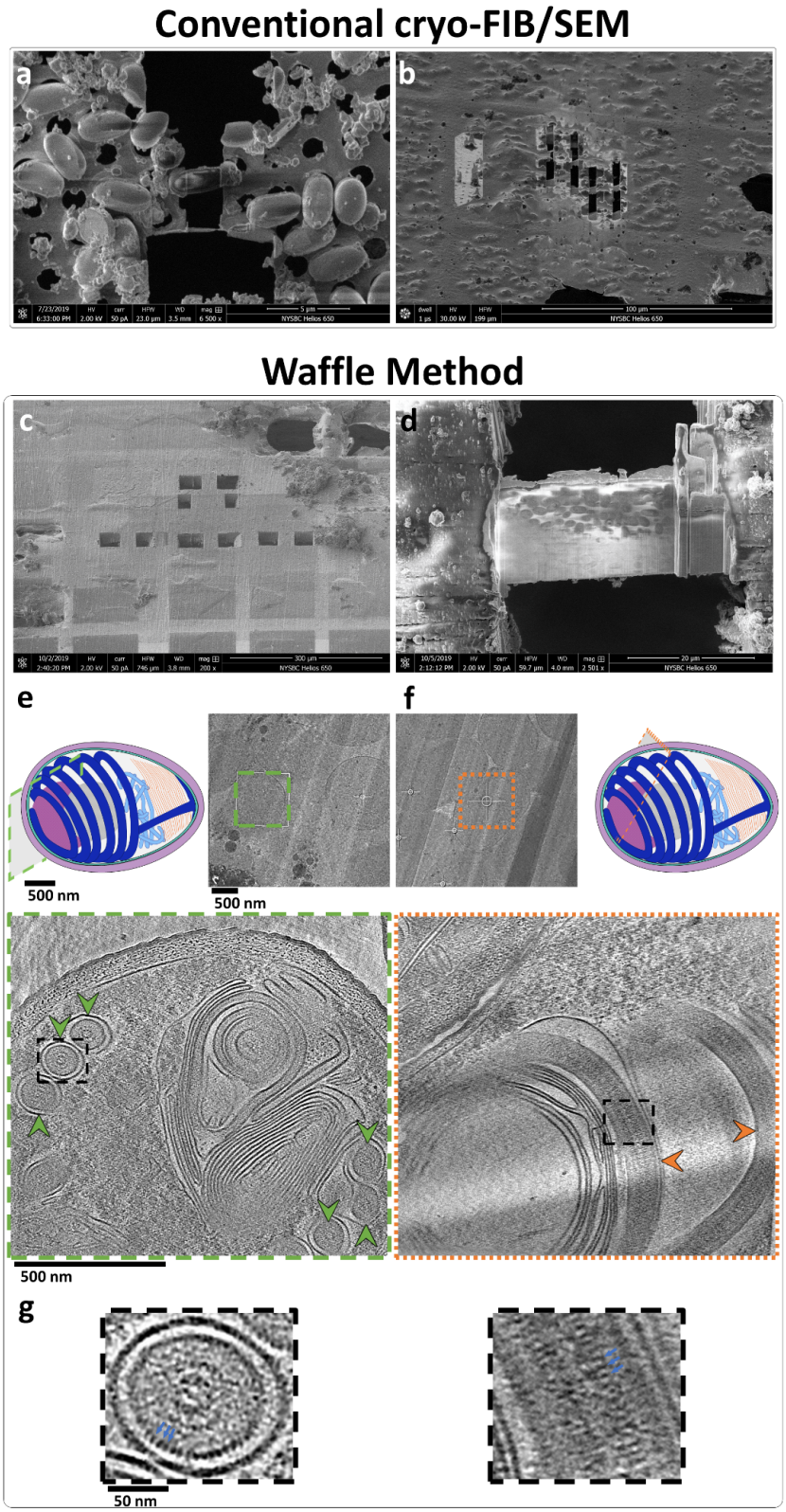

Figure 3 | Example of how the Waffle Method solves multiple problems of conventional cryo-FIB/SEM of microsporidian spores. **(a,b)** Small cells milled by conventional cryo-FIB/SEM where the samples were back-blotted and plunge frozen. The cells are individually-milled due to low concentration, leading to low-throughput. **(a)** SEM image of an individually-milled cell (~1.5 x 3 µm). **(b)** FIB image of several individually-milled cells. **(c-g)** Small cells prepared using the Waffle Method. **(c)** SEM image of a waffle with several trenches prepared. **(d)** SEM image of a completed waffle lamella (~30 x 20 µm) with a notch mill showing several orientations of the spores. **(e)** A low-mag TEM image of a waffled microsporidian spore lamella alongside a schematic diagram of a spore with the high-mag cryo-ET collection area approximated (green outlined cross-section). Below is a slice-through of the high-mag tomogram with arrows showing axial views of the polar tube in the spore (green arrows). The polar tube in the schematic diagram is colored dark blue, exhibiting a fixed orientation relative to the major axis of the spore. **(f)** A low-mag TEM image of a waffled microsporidian spore lamella alongside a schematic diagram of a spore with the high-mag cryo-ET collection area approximated (orange outlined cross-section). The spore cross-section is roughly orthogonal to the spore in (e), as the diagrams show. Below is a slice-through of the high-mag tomogram with arrows showing side views of the polar tube in the spore (orange arrows). **(g)** The dotted black line insets in (e) and (f) magnified by 4x highlighting the ~2.5 nm features on the second cylindrical layer (blue arrows). Tomogram slice-through videos are shown in Supplemental Video 3. (a,c-g) show *E. hellem* microsporidian spores while (b) shows *A. algerae* microsporidian spores.

Figure 4: Tomogram slice-throughs of four additional specimens

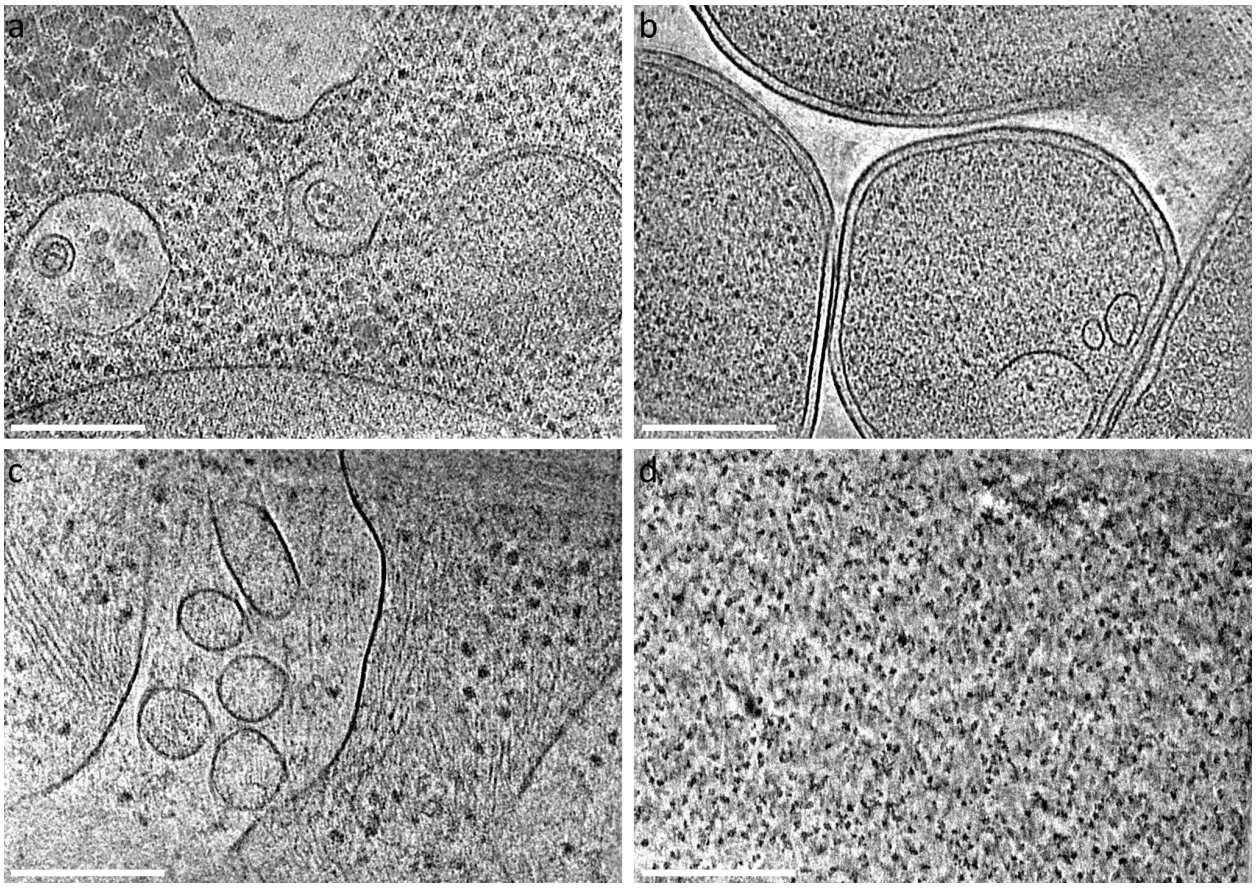

Figure 4 | Tomogram slice-throughs of yeast *S. cerevisiae* **(a)**, *E. coli* BL21 (DE3) + *Leviviridae* PP7-PP7 virus capsid proteins **(b)**, HEK 293S GnTI- **(c)**, and porcine thyroglobulin (ThG) single particle **(d)** waffle lamellae. Scale bars are 200 nm.

Figure 5: Potential additional uses for the Waffle Method

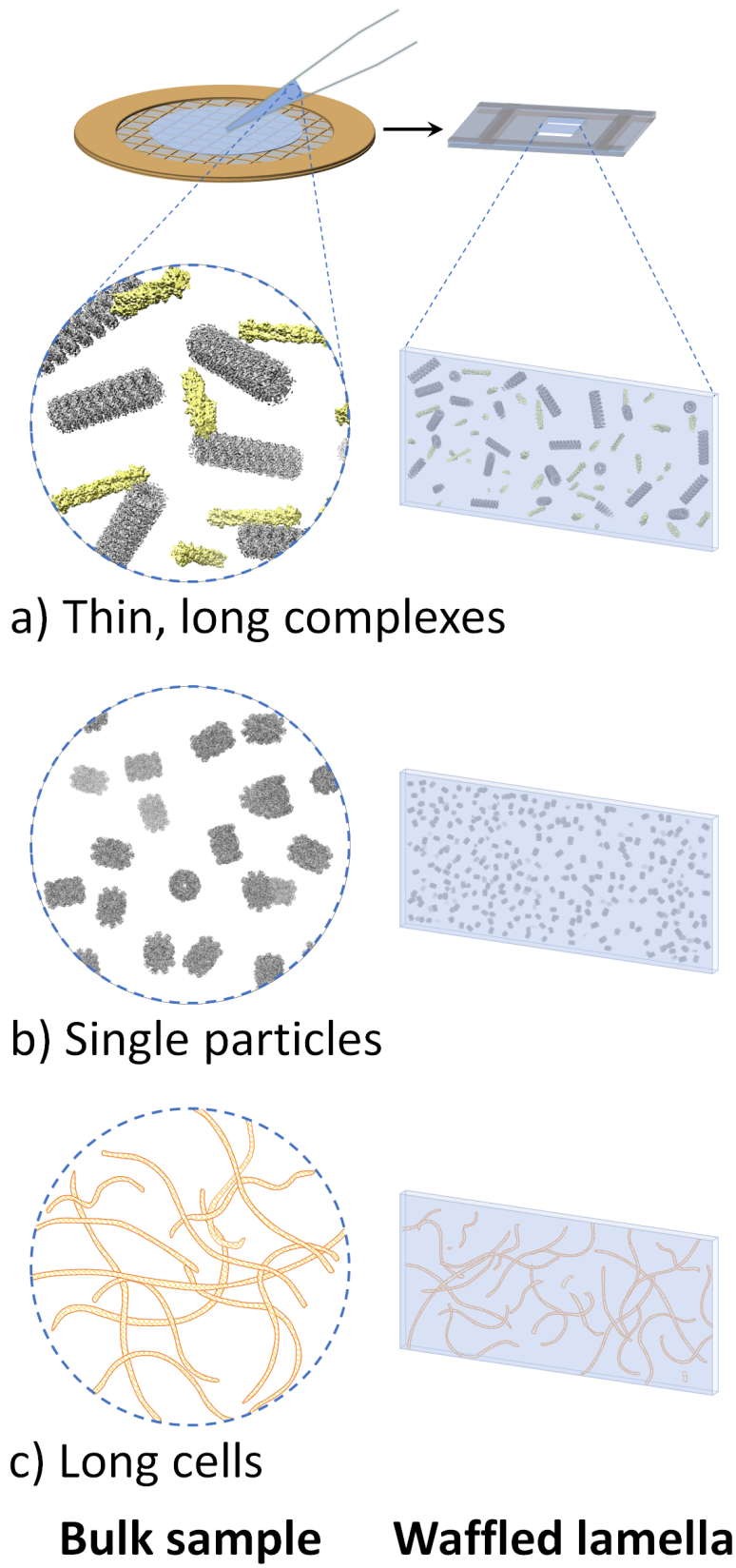

Figure 5 | Additional potential issues that the Waffle Method may rectify. **(a)** Thin, long complexes, such as actin and microtubules depicted here, will exist in all orientations in waffled lamellae. **(b)** Single particle protein samples that exhibit inhibitive issues when prepared conventionally in a thin film may be prepared without those issues using the Waffle Method. **(c)** Long cells, such as the *Spirochaeta plicatilis* depicted here (0.2 – 0.75 µm in diameter by 5 – 250 µm in length), may be imaged in all orientations with the Waffle Method, which may also allow for the cell membrane to be studied more thoroughly. UCSF Chimera[34] was used for molecule depiction in (a) and (b).

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Supplement

Supplemental Video 1: General Waffle Method workflow

https://drive.google.com/file/d/19rnSndFkiMCv7gH3QVTTzwIBeQb5Efy6/view?usp=sharing
or
https://nysbc-my.sharepoint.com/:v:/g/personal/anoble_nysbc_org/ETFptW-wHA1LkdLvvjgCtBcBmnmswMM3QcNkyJ-Qq7g1Pg?e=dbeP5S

Supplemental Video 1 | Animation showing the general Waffle Method workflow.

Supplemental Video 2: Kotaro making a waffle grid

https://drive.google.com/file/d/1raR-vAbS0LKzDDOt_dWiCpcaS8lA4JsX/view?usp=sharing
or
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Supplemental Video 2 | Video of Kotaro Kelley making a waffle grid. Here he performs most of Step 1 in Figure 1: *(0:11)* He places a planchette hat previously coated with 1-hexadecene into an HPF tip that was previously cleaned with ethanol, *(0:27)* several microliters of sample are applied to the planchette hat, *(0:47)* excess sample is wicked away after the top planchette hat is positioned, *(1:09)* the sample is high-pressure frozen, and *(1:34)* the waffled assembly is removed from the HPF tip in LN2.

Supplemental Video 3: Microsporidian spore tomograms

https://drive.google.com/file/d/1BayLz9zvmCitRLxvqOQhaw2_Y9VBmm8B/view?usp=sharing
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Supplemental Video 3 | Tomogram slice-through videos of the microsporidian spores shown in Figure 3.

Supplemental Video 4: Waffle milling time-lapse video

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Supplemental Video 4 | Time-lapse video of coarse-to-fine waffle milling using AutoTEM on the Aquilos 2.

Supplemental Video 5: Yeast tomograms

https://drive.google.com/file/d/1Z20fGGLV7OiX47QzOClnZieyd5QHvOzs/view?usp=sharing

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Supplemental Video 5 | Tomogram slice-through videos of the yeast *S. cerevisiae* cells. The left tomogram is 250 nm thick and the right tomogram is 500 nm thick.

Supplemental Video 6: *E. coli* tomograms

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or

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Supplemental Video 6 | Tomogram slice-through videos of the *E. coli* cells.

Supplemental Video 7: HEK 293S cell tomogram

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Supplemental Video 7 | Tomogram slice-through video of the HEK cells.

Supplemental Video 8: Low-magnification *E. coli* tomogram

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Supplemental Video 8 | Low magnification tomogram slice-through videos of the *E. coli* cells showing top-ends and sides of cell outer membranes.

Supplemental Video 9: Possible mechanism for notch milling success

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Supplemental Video 9 | Animation of the illustration in Supplemental Figure 7 of why notch milling waffled lamellae may allow for lamellae to withstand directional and angular forces applied to the grid.

Supplemental Video 10: Single particle ThG waffle tomograms

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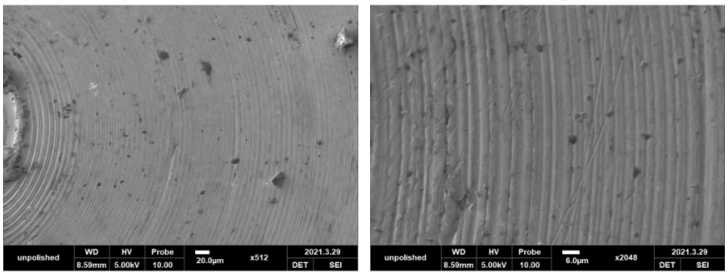
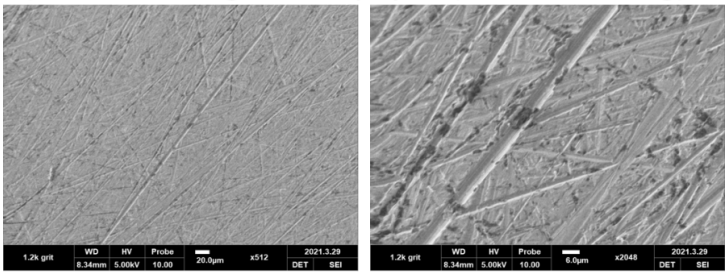
Supplemental Video 10 | Tomogram slice-through videos of the ThG single particle lamella.

Supplemental Figure 1: Planchette hat sanding

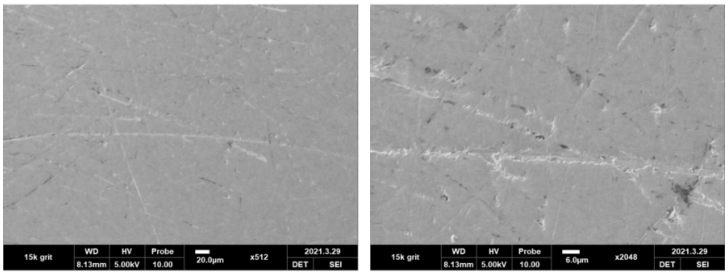
a) New planchette hat (500x, 2kx mag)



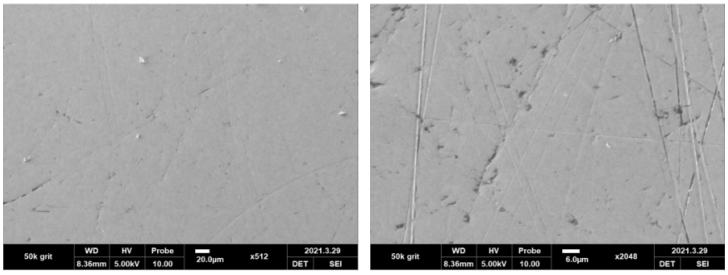
b) After 1,200 grit sandpaper (500x, 2kx mag)



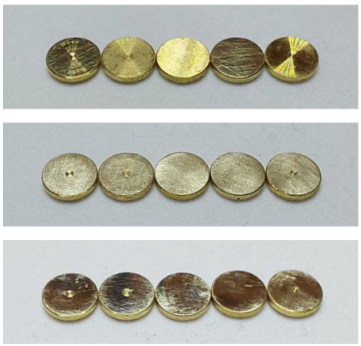
c) After 15,000 grit sandpaper (500x, 2kx mag)



d) After 50,000 grit sandpaper (500x, 2kx mag)



e)



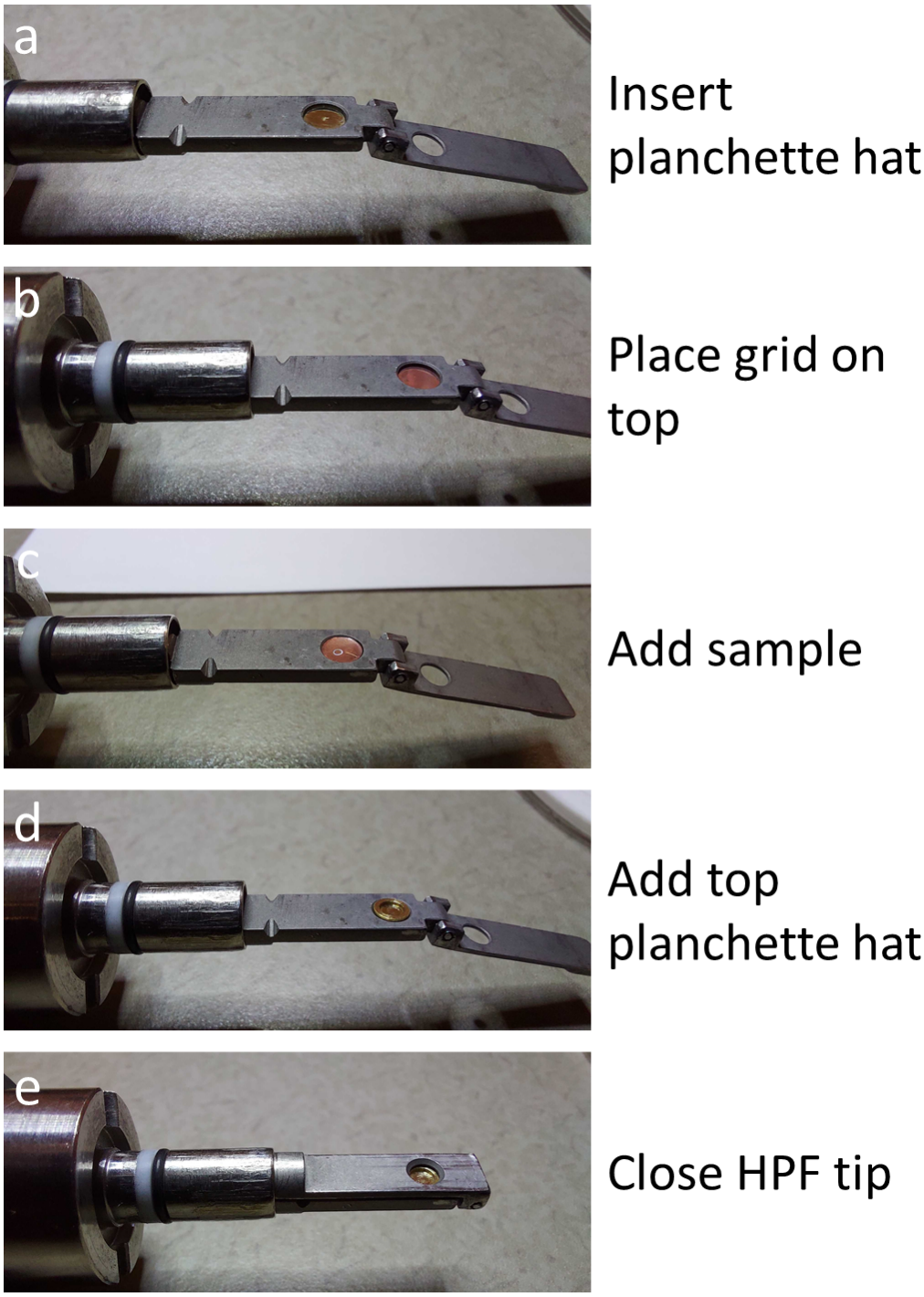
New planchette hats

After 1,200 grit sandpaper

After 15,000 grit sandpaper

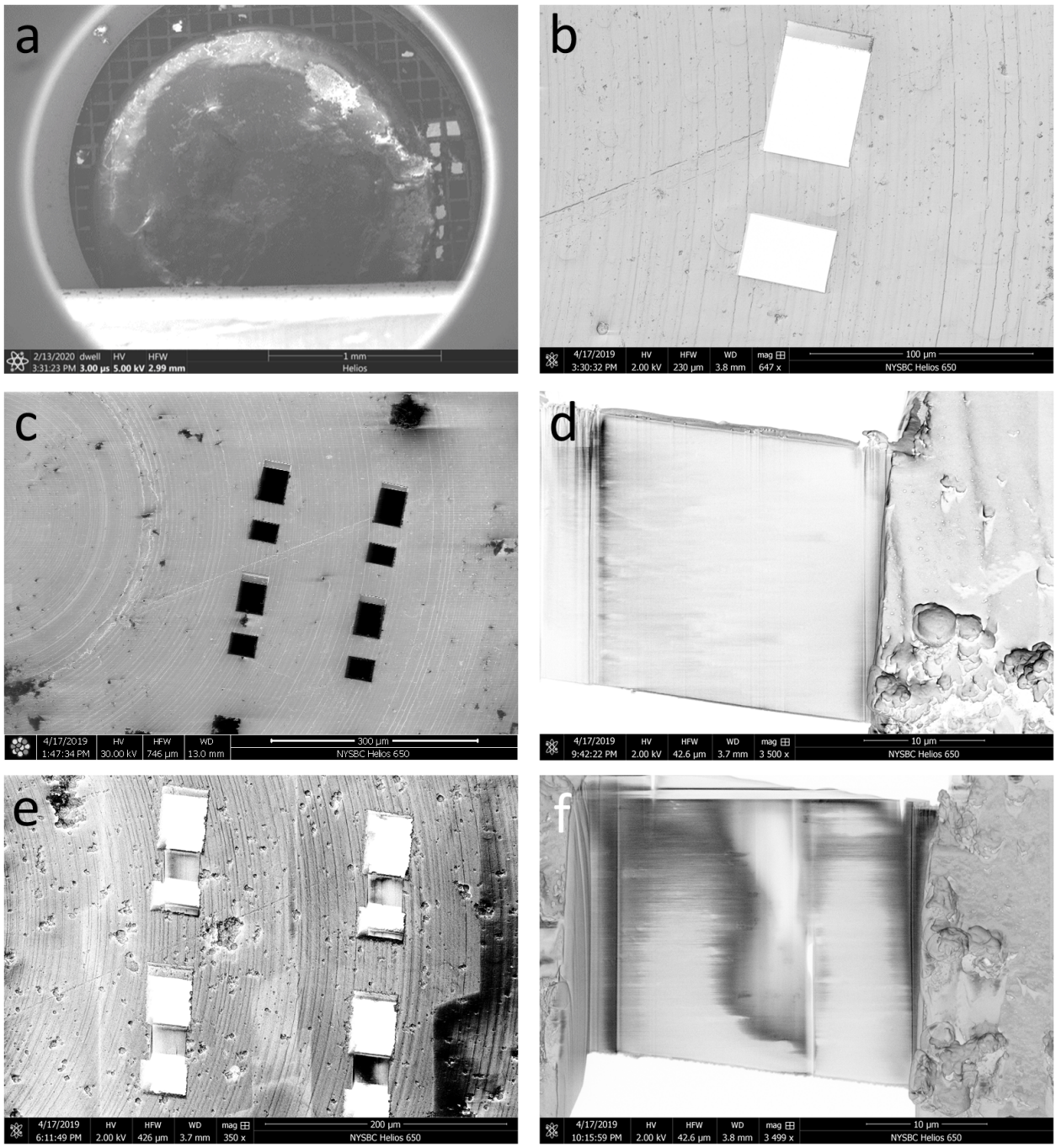
Supplemental Figure 1 | Images of a planchette hats at different sandpaper steps to illustrate surface smoothness. **(a)** SEM image of a section of a fresh planchette hat. 500x magnification image on the left and 2,000x magnification image of the center of the 500x image on the right. **(b)** After sanding with 1,200 grit sandpaper. **(c)** After additional sanding with 15,000 grit sandpaper. **(d)** After additional sanding with 50,000 grit sandpaper. **(e)** Low-magnification images of a series of planchette hats before sanding (top), after 1,200 grit sandpaper (middle), and after 15,000 grit sandpaper (bottom). Notice that the smoothness difference between (c) and (d) is negligible. SEM images were collected on a Cube II Mini-SEM (EMCRAFTS, Korea).

Supplemental Figure 2: Waffle assembly



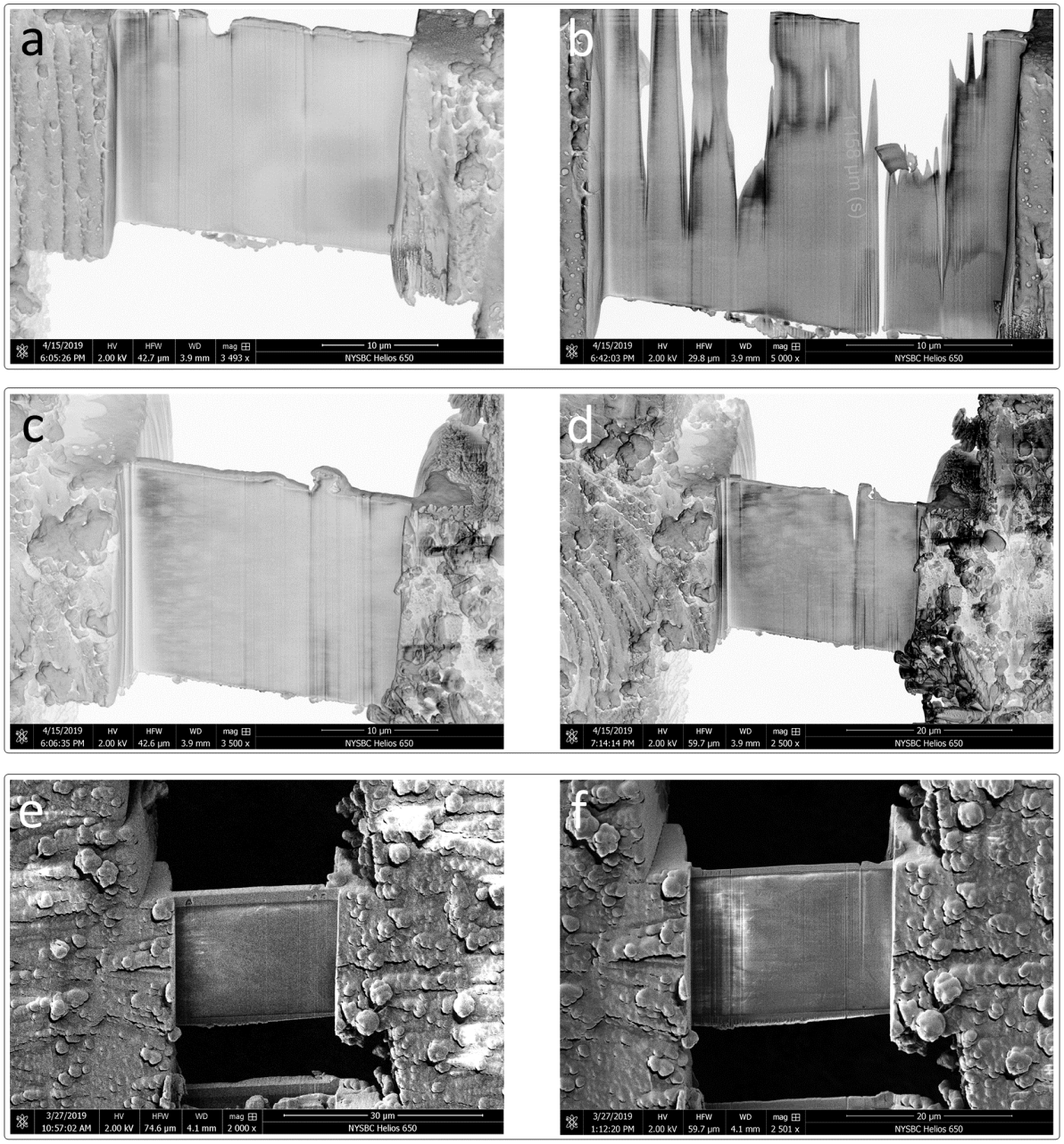
Supplemental Figure 2 | Images of the waffle assembly. Here, a waffle is assembled inside of a Wohlwend HPF tip. First a polished planchette hat with a fresh coat of 1-hexadecane is placed in the freshly-cleaned HPF tip **(a)**, then a freshly glow-discharged EM grid with 25 nm of extra carbon is placed on top with the grid bar side facing up **(b)**, several µL of sample is added on top of the grid **(c)**, the top polished planchette hat is added to the top **(d)**, and the HPF tip is closed before freezing **(e)**. Excess sample may need to be removed prior to freezing.

Supplemental Figure 3: Waffle milling workflow



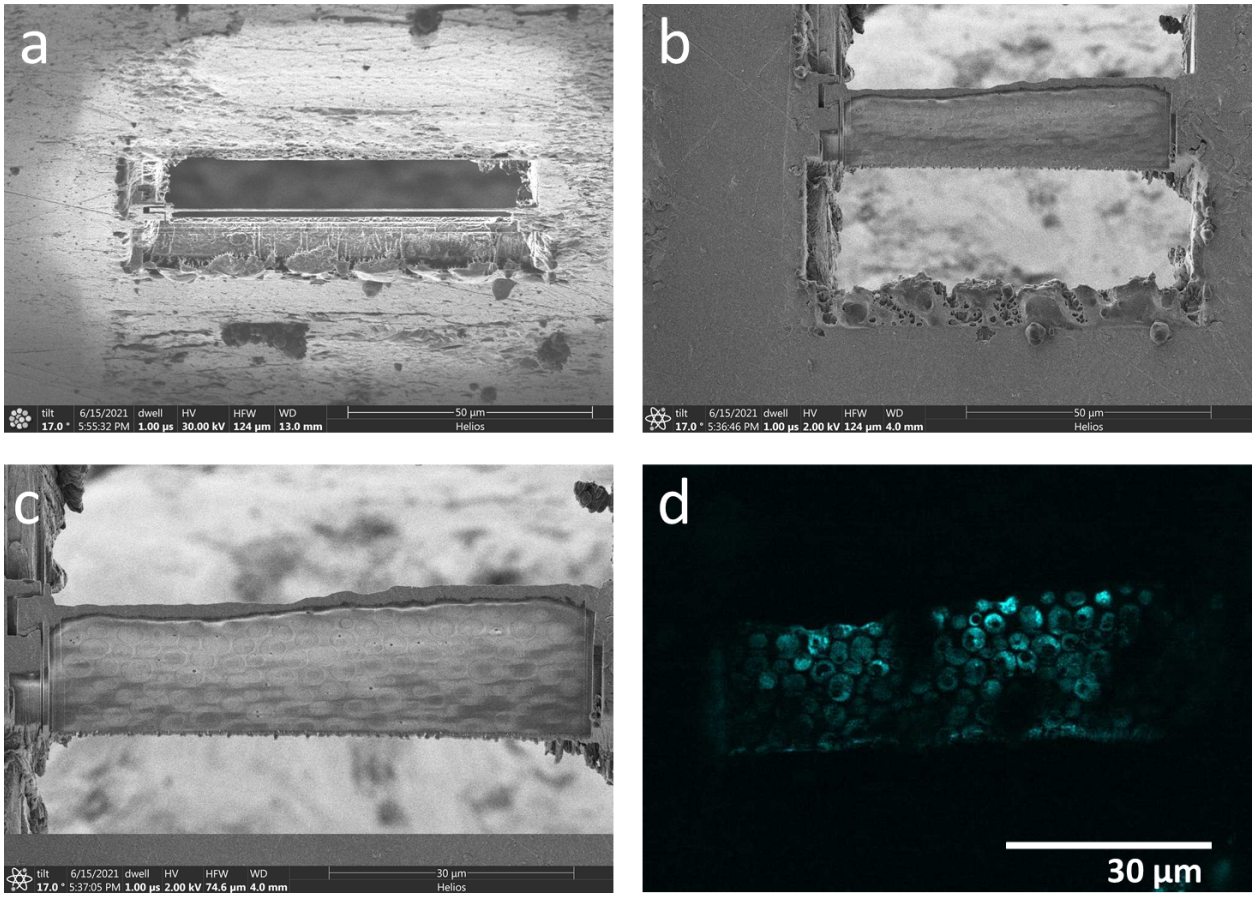
Supplemental Figure 3 | FIB/SEM images of the waffle milling workflow. **(a)** After creating a waffled sample, **(b,c)** two trenches per area of interest are milled as perpendicular to the plane of the grid as possible and tens of microns apart. **(d,e)** The grid is then tilted to the desired angle and milled first coarsely then finely while keeping the waffle platinum layer on the leading edge of milling. **(f)** The resulting lamellae are then polished. Notches may be milled for stress-relief between (c) and (d), as shown in Figure 2.

Supplemental Figure 4: Importance of sufficiently smooth waffles and a sufficient amount of platinum GIS pre-coating



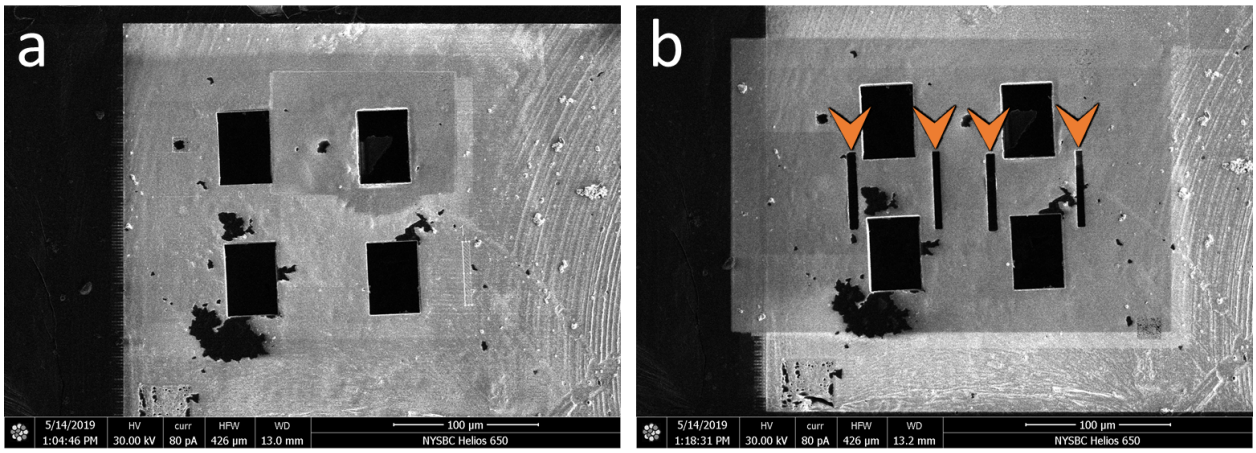
Supplemental Figure 4 | SEM images from three separate milling areas showing the importance of sufficiently smooth waffles and applying a sufficient amount of platinum GIS pre-coating. **(a)** shows a lamella milled to several hundred nanometers thickness with an insufficient amount of insufficiently smooth platinum GIS pre-coating causing further milling to curtain and deteriorate the lamella **(b)**. **(c)** shows a lamella milled to several hundred nanometers thickness with sufficiently smooth platinum GIS on the left side of the lamella, but not on the right. Finer milling results in reduced curtaining on the left side compared to the right **(d)**. **(e)** shows a lamella milled to several hundred nanometers thick with a sufficient amount of sufficiently smooth platinum GIS. Finer milling results in minimal curtaining **(f)**.

Supplemental Figure 5: Very wide yeast waffle lamella



Supplemental Figure 5 | FIB/SEM images of waffle milling of very wide waffle lamella of yeast *S. cerevisiae* with a notch milled into the left-hand side. This lamella is about 70 x 25 µm. **(a)** FIB image of the finished waffle lamella. **(b)** SEM image of finished lamella. **(c)** Zoomed-in SEM image of the lamella in (b) showing dozens of imageable yeast cell cross-sections. **(d)** Zeiss Airyscan 2 cryo-FLM image (Zeiss, Oberkochen, Germany) of the lamella after cryo-ET collection showing GFP-tags.

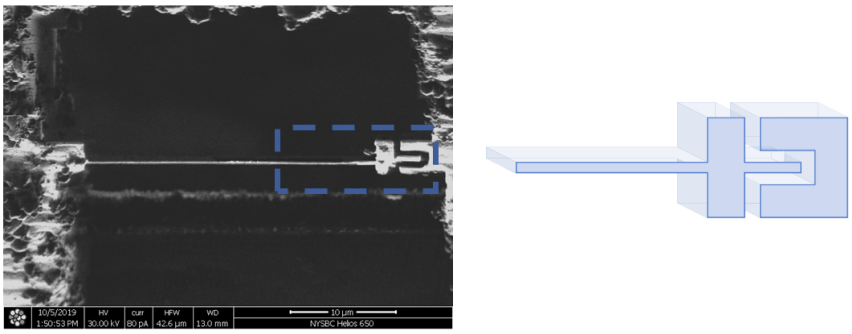
Supplemental Figure 6: Gap milling development for waffled lamellae



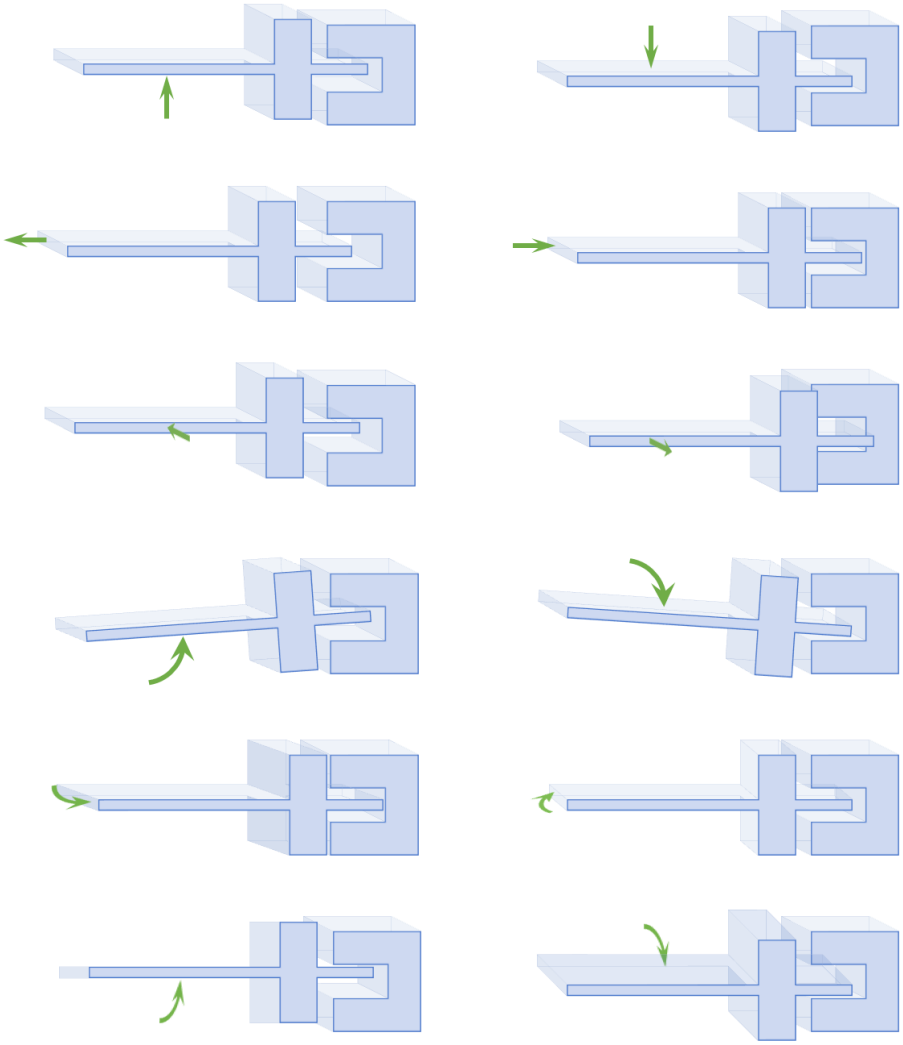
Supplemental Figure 6 | Initial stress-relief gap milling development for waffled lamellae. **(a)** Two trench-milled slabs. **(b)** Gaps (orange arrows) about 6 µm in width on either side of the slabs intended for lamellae stress-relief. Milling gaps in the sides of the lamellae after coarse milling was also explored (not pictured here). Neither method proved to relieve stress enough to avoid damage to waffled lamellae.

Supplemental Figure 7: Possible mechanism for notch milling success

a) After milling

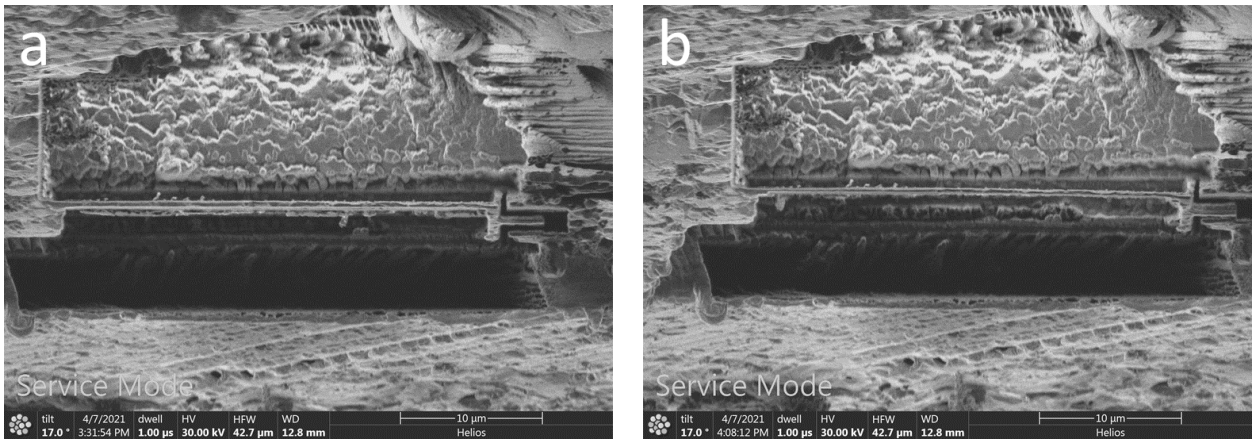


b) During applied forces on grid



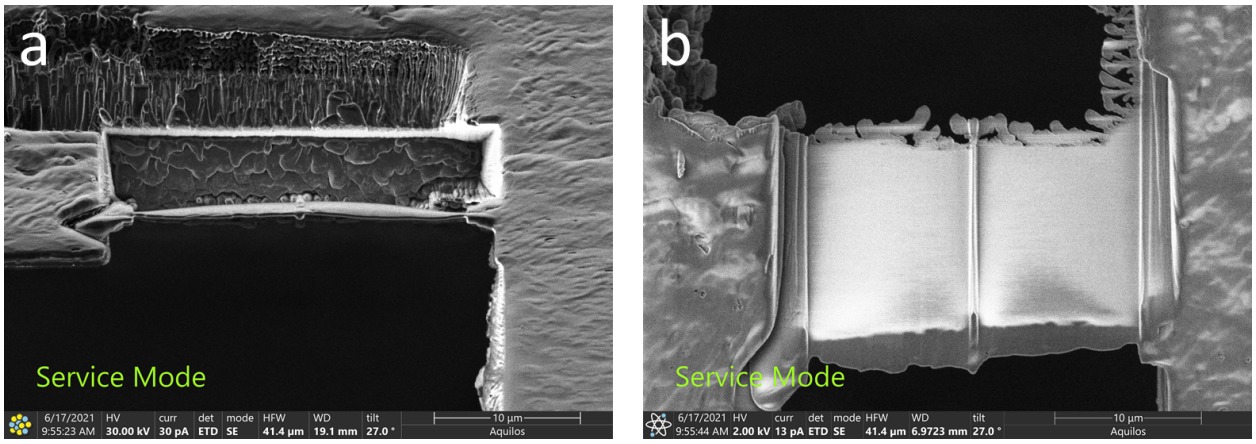
Supplemental Figure 7 | Illustrations of why notch milling waffled lamellae may allow for lamellae to withstand directional and angular forces applied to the grid. **(a)** Immediately after notch milling, the lamellae tab is separated from the notch. **(b)** Directional and angular forces applied during grid handling and from thermal fluctuations are dissipated by the notches instead of affecting the lamellae.

Supplemental Figure 8: Notch resting



Supplemental Figure 8 | FIB images a milled notch coming to rest. **(a)** Image of a waffle lamella part way through milling and after the notch has already been milled. **(b)** Image of the same lamella after about 30 minutes had passed. No milling operations were performed on the lamella between (a) and (b).

Supplemental Figure 9: Arrowhead notch



Supplemental Figure 9 | FIB image **(a)** and SEM image **(b)** of an alternative arrowhead notch (on the left side of the lamella).