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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 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 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. 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.

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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[7,8]. 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[9]. Recently, cryo-microtomy and cryo-FIB-mill trenching of vitrified *C. elegans in-situ* followed by micromanipulator liftout and further FIB-milling on custom EM grids has been demonstrated[10,11]. 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[11].

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) multiple very large (greater than 10 x 20 µm) and specimen-dense lamellae may be prepared regardless of the specimen size, which substantially increases throughput. Moreover, the waffle

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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[12,13]. The object of interest within the dormant microsporidian spores - the polar tube - is in a fixed orientation relative to the spore[13] and the spores exhibit preferred orientation on the grid, constituting an intractable issue for conventional cryo-FIB/SEM. We show that the waffle method solves the preferred orientation issues 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.

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.[14–16]; 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 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 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 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

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Kotaro's independent exploration of stress-relief gaps for lamella. This manuscript serves as a time capsule of his work in these areas. The methods and the results are presented here as-is while we work to re-discover and re-build many of the practical nuances of the waffle method, which we anticipate will result in an updated and more complete manuscript in the future.

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Results

Overall workflow

Figure 1 and Supplemental Video 1 show the overall waffle method workflow. Before making the waffle, the hardware needs to be prepared (Step 0): a) planchette hats should be polished 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 Video 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-CLEM.

The waffle grid is then transferred to a flat cryo-FIB/SEM grid holder 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 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 step-wise 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 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

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Figure 1 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 (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: 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, 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 or camera for loading grids in proper orientations.
5) A flat cryo-FIB/SEM grid holder.
6) Cryo-CLEM system for screening waffles before milling and analyzing waffled lamellae after milling and/or after cryo-ET collection.
7) Fluorescently-tagged regions of interest in the specimen for CLEM identification.

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Waffle method applied to small specimens with preferred orientation issues

To exemplify several of the advantages of the waffle method, we applied it to dormant microsporidian *Encephalitozoon hellem* spores. *E. hellem* spores are about 1.5 µm in diameter and 2 – 4 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[13] (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). 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

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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-CLEM loading/unloading, cryo-TEM loading). To rigidify the grid, about 25 nm of carbon is sputtered onto the non-grid bar side.

The planchette hats and optional spacer ring are coated with 1-hexadecene to allow for easier disassembly of the waffle after freezing[17,18]. The HPF tip is cleaned with ethanol. The waffle grid is assembled in the HPF tip as shown in Supplemental Video 2: A few 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.

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Analysis of waffled microsporidian spores

Figure 3d shows a waffled microsporidian spore lamella in the SEM. The lamella is about 30 µm by 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 2). 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.

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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 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. Other directionally-oriented organelles inside the microsporidia may also be studied as a result. Several developments and implementations were key to the success of the development of the waffle method. Four key components that resolve the critical issue of lamella curtaining are 1) polishing planchette hats which substantially smooths the initial waffle surface, 2) creating sufficiently smooth waffles, 3) depositing a sufficient amount of platinum GIS prior to FIB-milling to protect the lamella during milling (Supplemental Figure 2), and 4) consistently milling lamella with the FIB beam initially intersecting the platinum GIS coat. Other key components for the waffle method workflow include: the additional ~25 nm carbon coat on the EM grid strengthens the substrate to help reduce broken squares, using 1-hexadecene to allow the planchette hats and optional spacer to be more easily separated after high-pressure freezing, milling lamellae in parallel to reduce lamellae contamination, and notch milling to substantially increase the likelihood of lamellae surviving grid transfers.

Notch milling in particular proved to be a critical component during waffle method development. Initially, gap milling similar to Wolff et al.[19] was independently developed and tested prior to any publications (Supplemental Figure 3), 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

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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 4). 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. In our experience, this support is sufficient for lamellae stability during cryo-ET tilt-series collection.

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.[17]
- 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 stress because additional empty squares on the grid absorb more stress, thus reducing stress on the lamellae.
- 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/SEMs during fine milling[10]. 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.

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- 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 Video 2.
- A cryo-CLEM 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 4 depicts three potential applications of the waffle method. Long complexes like filaments and microtubules may be studied from all directions (Figure 4a), as opposed to in conventional single particle preparation where axial views do not exist. 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.[14–16] (Figure 4b). Long, skinny cells, such as the *Spirochaeta plicatilis* depicted in Figure 4c, 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.

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,

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there will be cross-sections of cell membranes in waffles, 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. 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. Less operator specialization is required. Higher throughput may be achieved; more lamellae can be made per grid. Finally, the waffle method is more automatable due to all cryo-FIB/SEM operations being performed on one grid.

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 will 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. 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. 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 automated to further increase throughput.

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Methods

Waffle grid preparation

(The following methods are accurate to the best of our current knowledge.)

The general waffle method workflow is described in the Results section. Here we describe the specific equipment and workflows used during waffle milling development. 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 are polished for several minutes using POL Metallpflege metal-polish and 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% O2 gas and 20% H2 gas for 30 seconds.

The HPF tip is cleaned prior to assembling the bottom of the waffle in the HPF tip as shown in Supplemental Video 2. A few 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 Bal-Tec HPM 010 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 and flat screwdrivers 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 a Quorum PP3000T prep chamber (Quorum Technologies, Great Britain) attached to an FEI Helios NanoLab 650 cryo-FIB/SEM (FEI, Hillsboro, OR). 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

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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. 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.

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. An FEI Helios NanoLab 650 was used to perform cryoFIB/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°).

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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[20]. *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.

Cryo-ET collection

Tilt-series were collected with Leginon[21] using an FEI Titan Krios using counting mode on a Gatan K2 BioQuantum with the energy filter slit width set to 20 eV. 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.

Cryo-ET processing

Tilt images were frame aligned with MotionCor2[22] without patches or dose weighting. Frame aligned tilt images were used for fiducial-less tilt-series alignment in Appion-Protomo[23–25]. 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[26] prior to reconstruction with Tomo3D[27,28] SIRT, then denoised using the Topaz-Denoise[29] pre-trained model. Tomograms were visualized with IMOD[30].

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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, we present here as much knowledge and data 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 this version of the manuscript, apart from Supplemental Figure 1a, were all performed by Kotaro. We are continuing to develop the waffle method and extensions of it as described in the Discussion. We hope his knowledge and spirit continue to make positive impacts on people's lives through biomedical research that extend beyond and are derivatives of his research.

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Author contributions

K.K. conceived of this project, developed and implemented the waffle method, and prepared and collected the waffle cryo-FIB/SEM-ET data. K.K. independently conceived and tested gap milling methods. K.K. and A.J.N. processed and analyzed the waffle data. A.J.N. prepared, collected, and analyzed the conventional cryo-FIB/SEM data. P.J. prepared the microsporidian spore samples. A.R. prepared and collected Supplemental Figure 1a. K.K., P.J., A.R., E.E., G.B., C.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. P.J., A.R., E.E., G.B., C.P., B.C., and A.J.N. edited the manuscript.

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Figure 1: General waffle method workflow

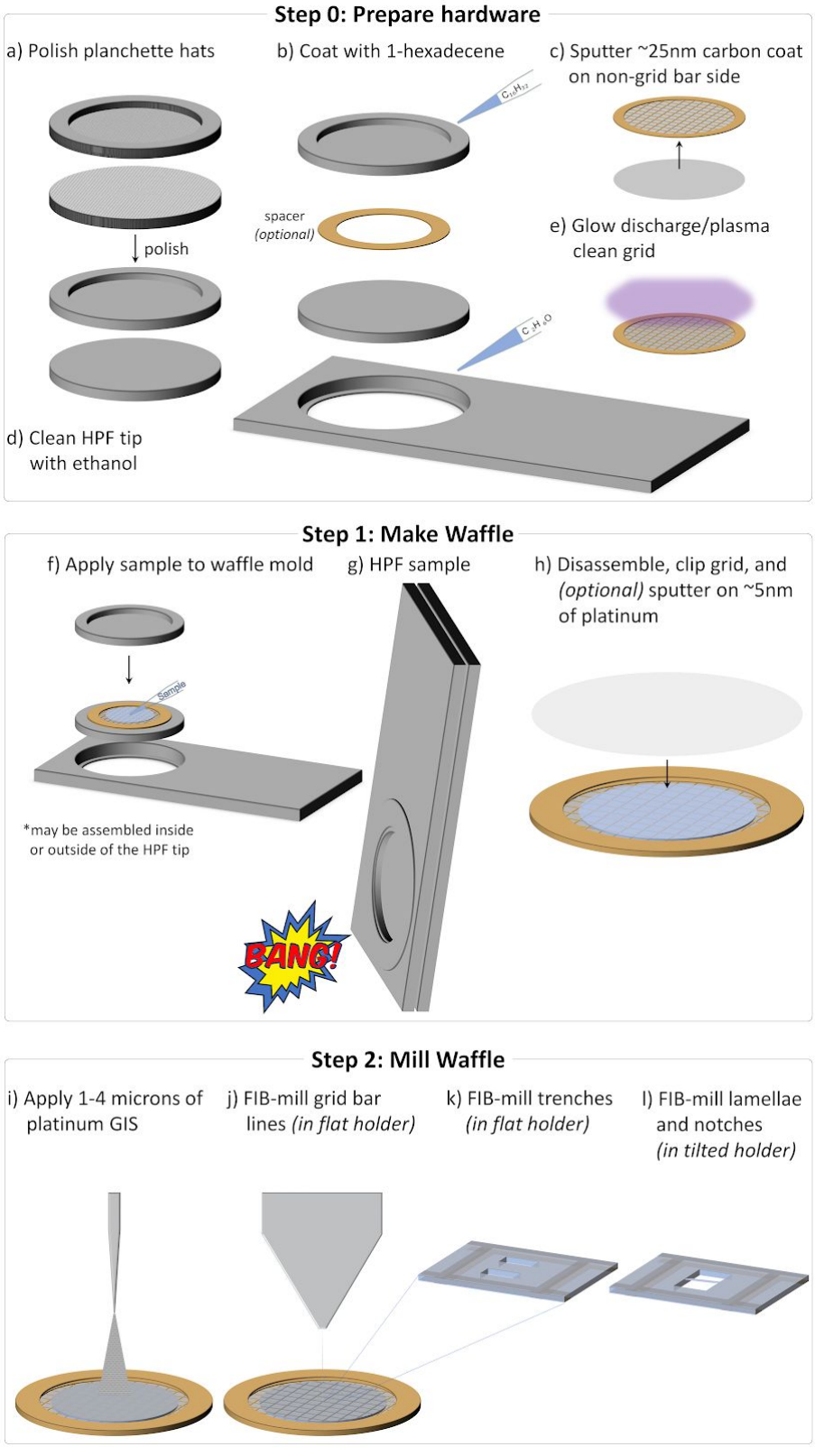

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Figure 1 | Schematic of the general workflow for creating a waffle grid. Supplemental Video 1 shows an animation of all steps. In Step 0, the waffle is prepared: **(a)** The planchette hats are polished to reduce lamellae curtaining, **(b)** The planchette 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-CLEM 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 1 shows waffle milling FIB/SEM images. Supplemental Video 2 shows most of Step 1 being performed.

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Figure 2: Notch milling for waffle lamellae stress-relief

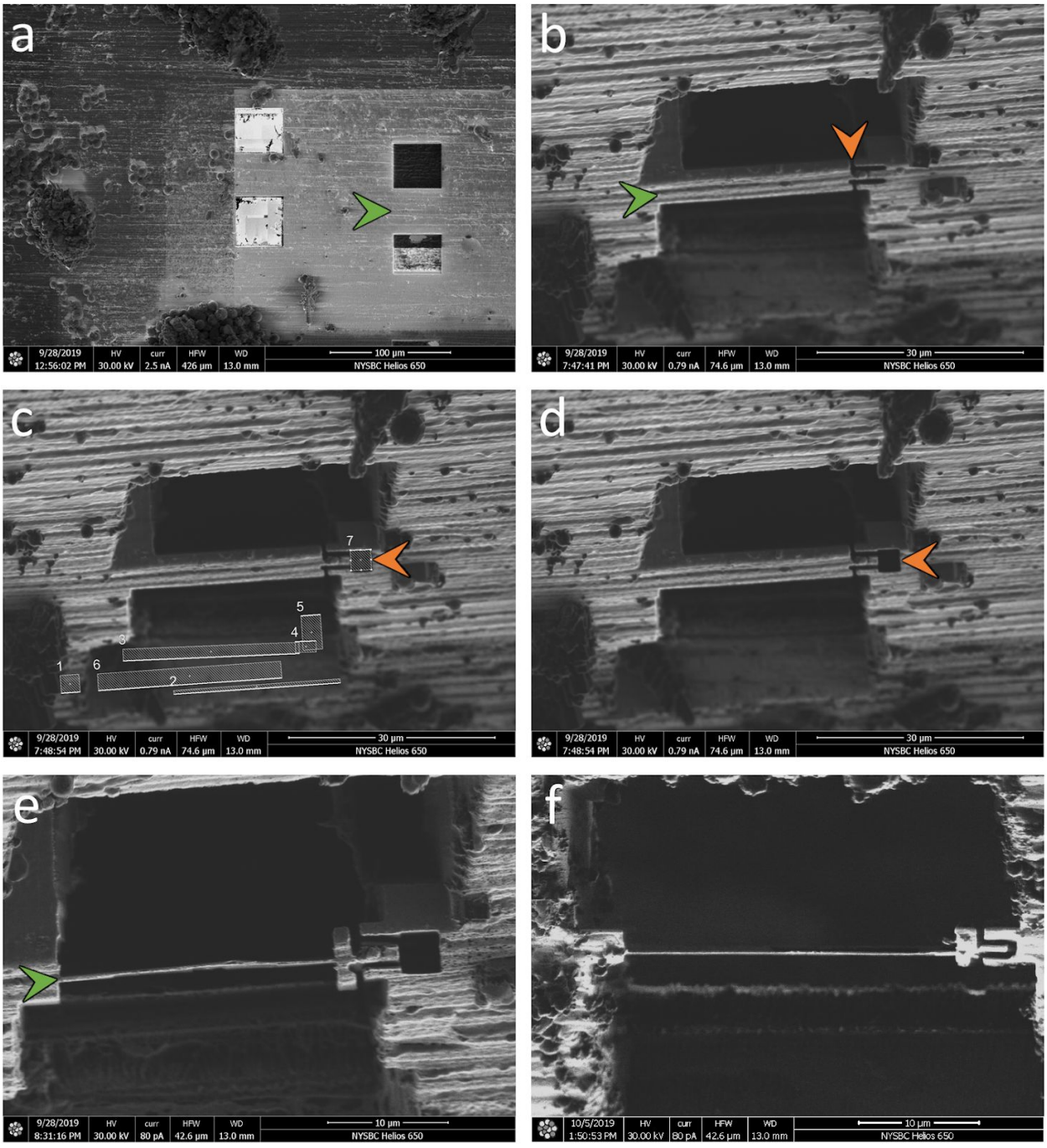

Figure 2 | FIB/SEM images of the notch milling workflow. After trench-milling **(a)** (green arrow), the resulting slab is coarsely milled down to <10 µm thick **(b)** (green arrow). The initial, incomplete notch design is milled into one side of the slab, with a segment still connected **(b)** (orange 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

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notch mill is presented in **(f)**. To allow for freedom of movement (see Supplemental Figure 4), each notch mill should separate the tab from the slab by several hundred nanometers.

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

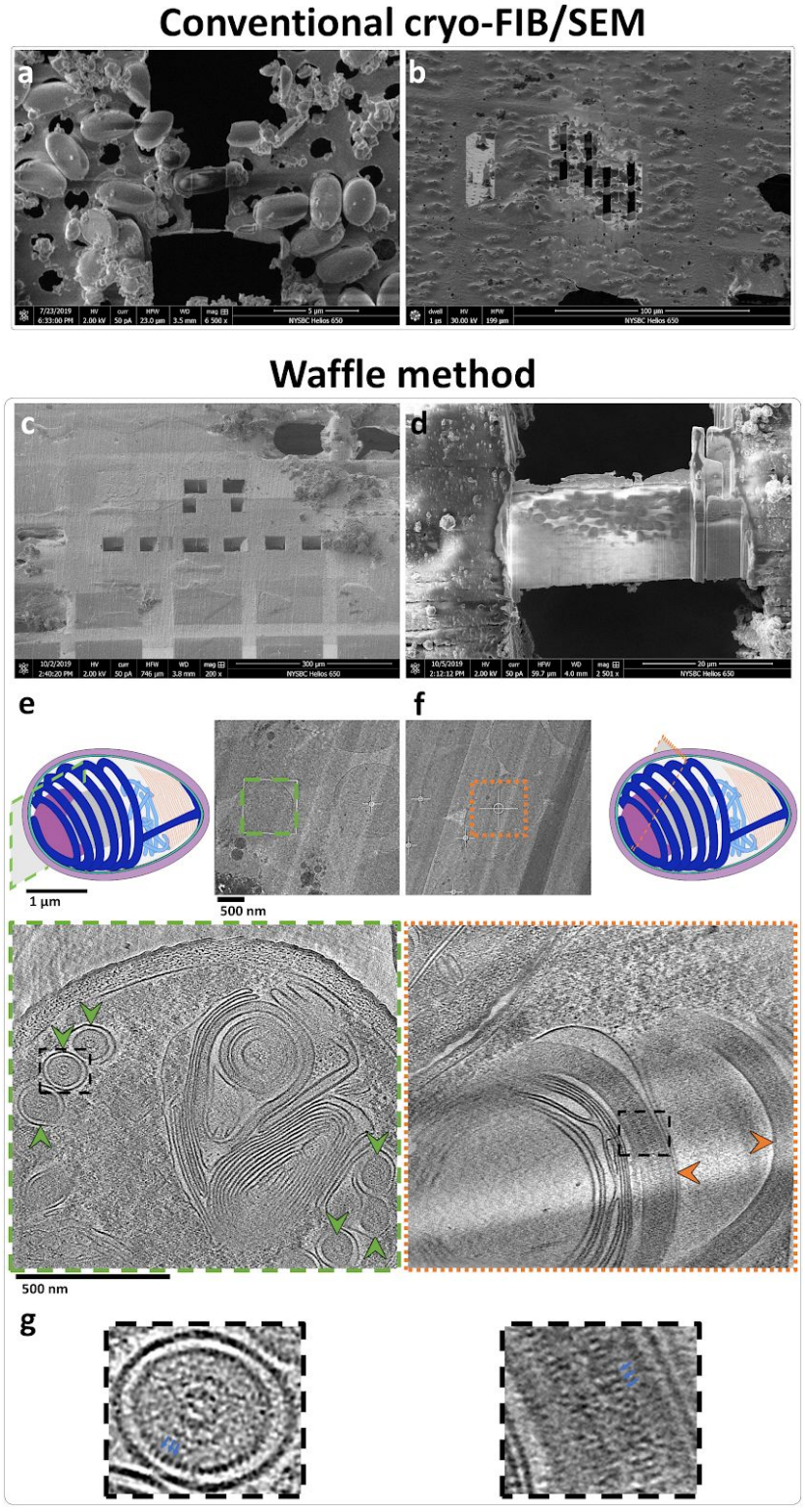

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Figure 3 | Example of how the waffle method solves multiple problems of conventional cryo-FIB/SEM of microsporidian spores. **(a,b)** Small cells milled by conventional cryo-FIB/SEM where the samples were back-blotted and plunge frozen. The cells are 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.

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Figure 4: Potential additional uses for the waffle method

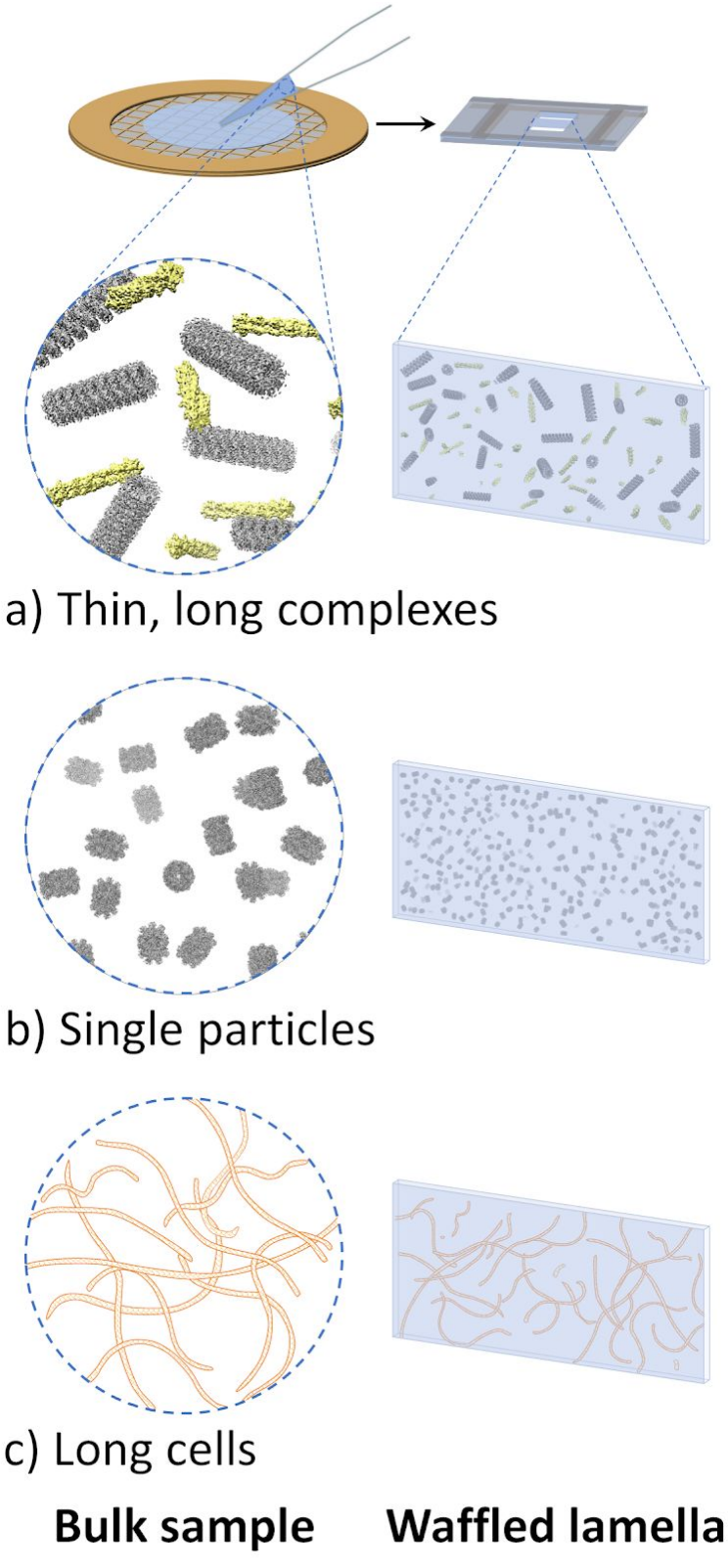

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Figure 4 | Additional potential issues that the waffle method may rectify. **(a)** Thin, long complexes, such as actin and microtubules shown here, will exist in all orientations in waffled lamellae. **(b)** Single particle protein samples that exhibit 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[31] 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.

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Supplemental Video 2: Kotaro making a waffle grid

https://drive.google.com/file/d/1raR-vAbS0LKzDDOt_dWiCpcaS8lA4JsX/view?usp=sharing

or

https://nysbc-my.sharepoint.com/:v:/g/personal/anoble_nysbc_org/ERYcAu_ky_5Km_-fvU7PtBMBm6W5N9I4EkLcxQZclF55Bg?e=irjatF

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 HPT tip that was previously cleaned with ethanol, (0:27) a few 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.

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Supplemental Video 3: Microsporidian spore tomograms

https://drive.google.com/file/d/1BayLz9zvmCitRLxvqOQhaw2_Y9VBmm8B/view?usp=sharing

or

https://nysbc-my.sharepoint.com/:v:/g/personal/anoble_nysbc_org/Ebva8-_3_JtNmcQbc6droToBEVMlKdOy4S1n4KjEPMipXg?e=p0RD7Q

Supplemental Video 3 | Tomogram slice-through videos of the microsporidian spores shown in Figure 3.

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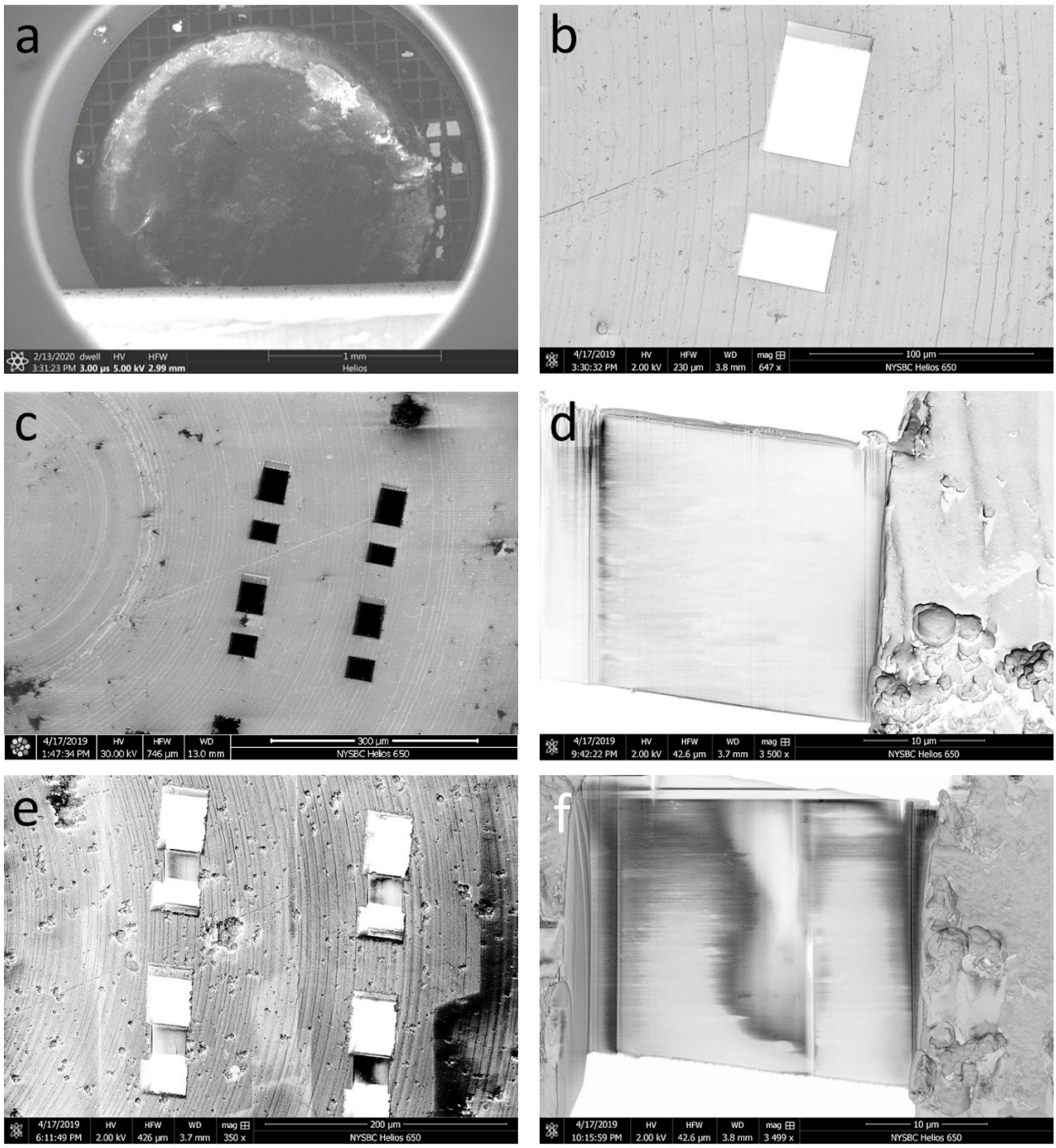
Supplemental Figure 1: Waffle milling workflow



Supplemental Figure 1 | 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.

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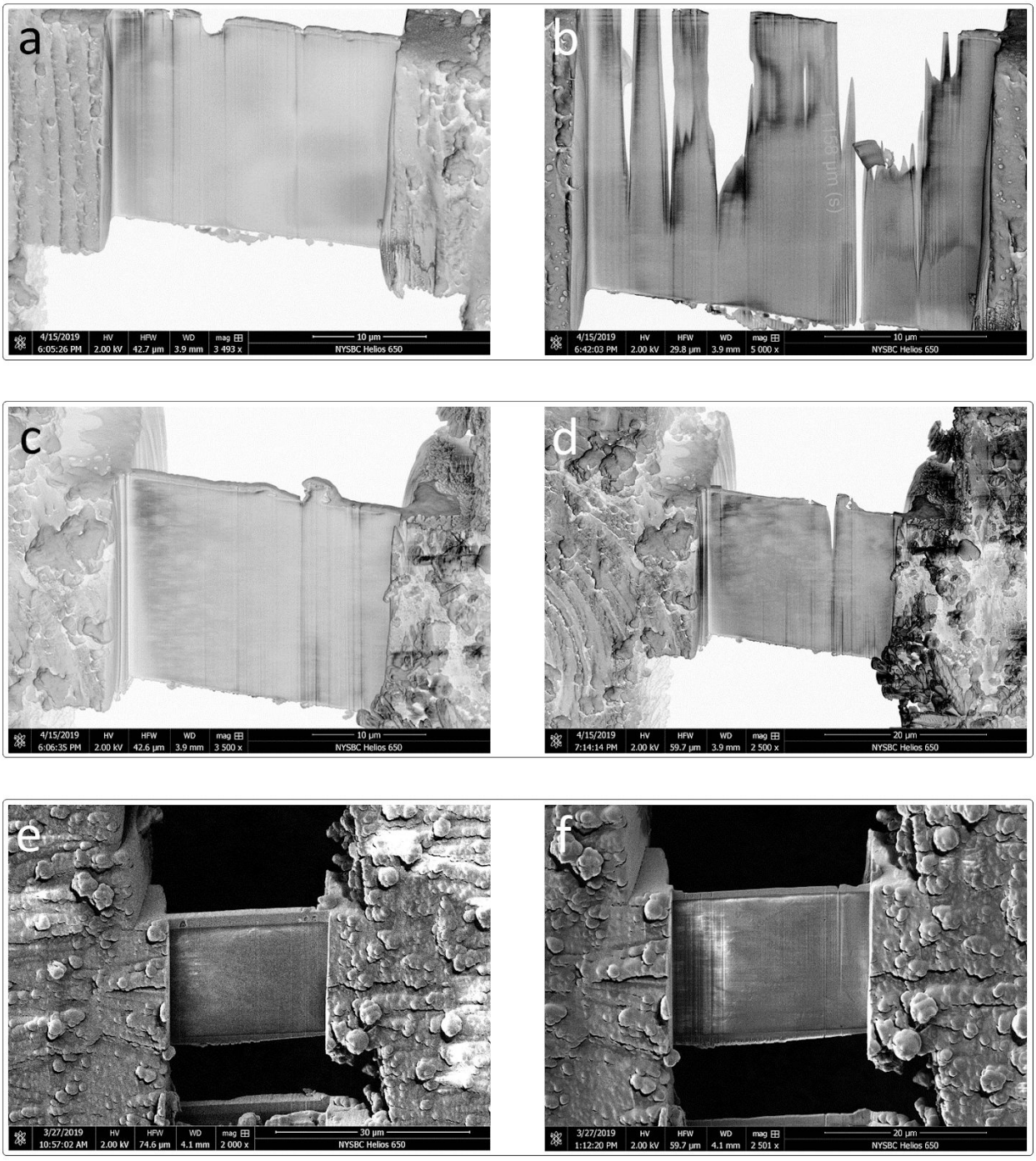
Supplemental Figure 2: Importance of sufficiently smooth waffles and a sufficient amount of platinum GIS pre-coating



Supplemental Figure 2 | 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)**

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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 very little curtaining on the left side compared to the right **(d)**. **(e)** shows a lamella milled to several hundred nanometers thickness with a sufficient amount of sufficiently smooth platinum GIS. Finer milling results in minimal curtaining **(f)**.

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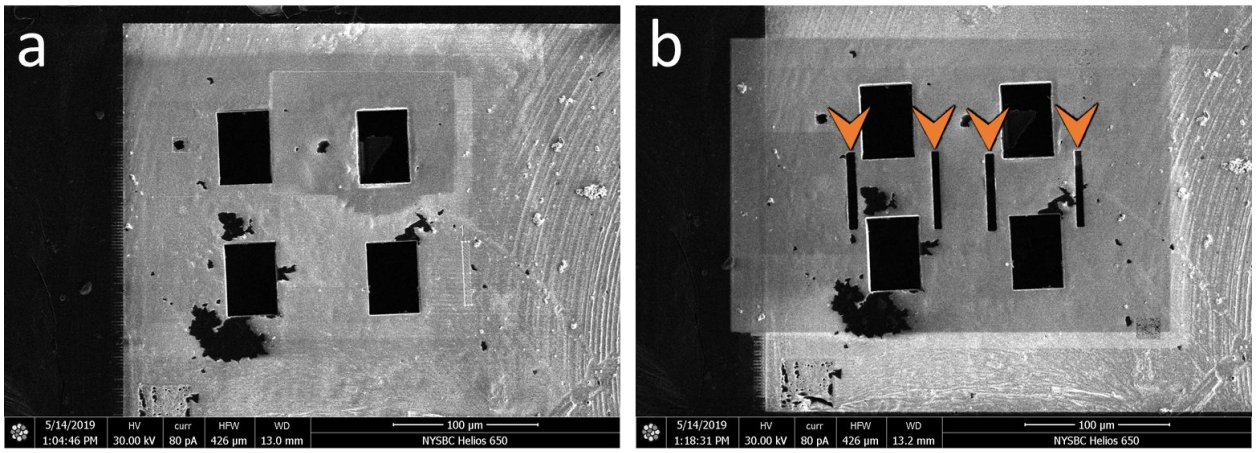
Supplemental Figure 3: Gap milling development for waffled lamellae



Supplemental Figure 3 | 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.

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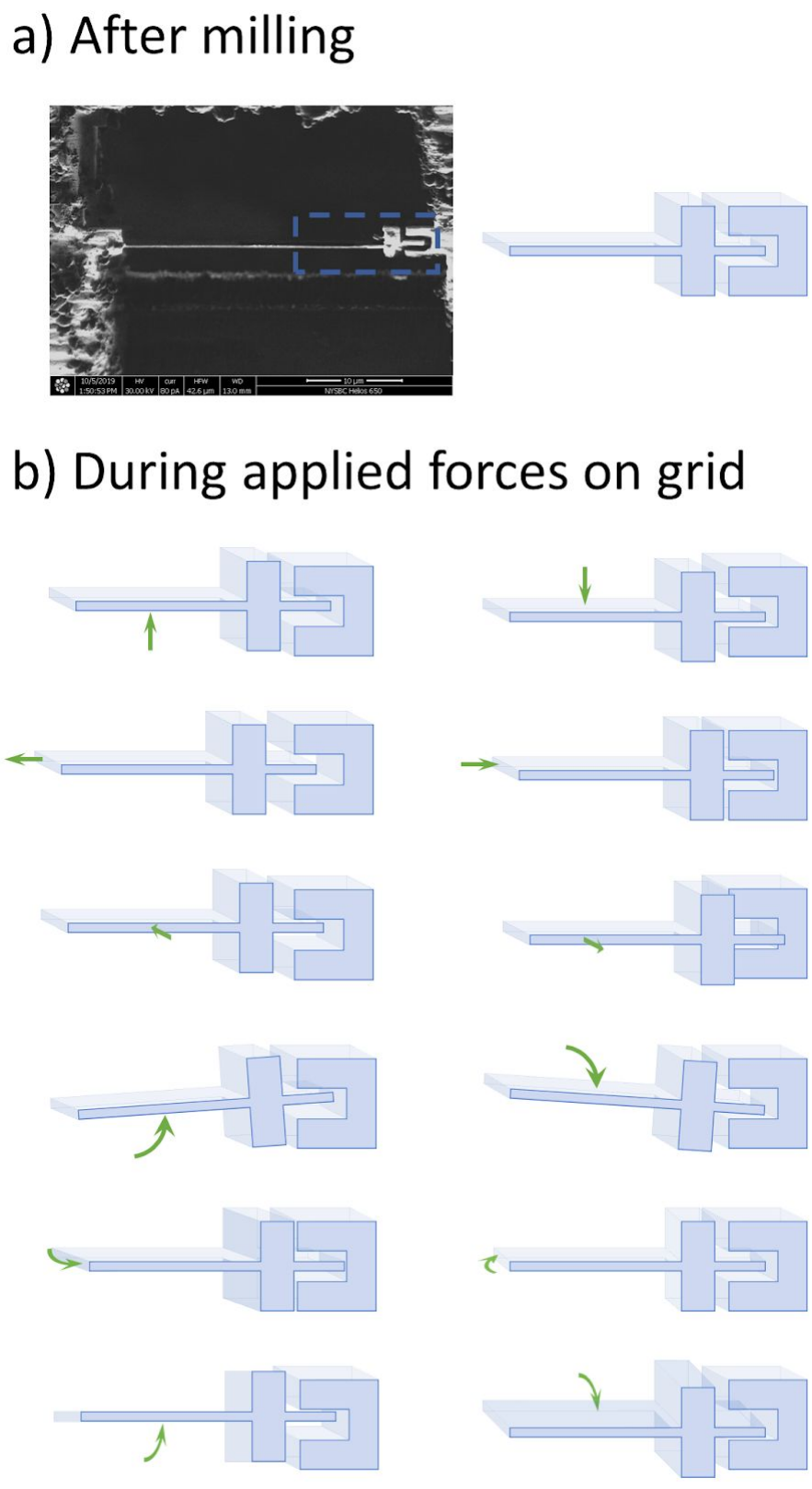
Supplemental Figure 4: Possible mechanism for notch milling success



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Supplemental Figure 4 | 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.