Cryo-EM Lamella Thickness: A Method Comparison

Penelope Paredes[1], Evan Reetz[1], Xueting Zhao[2], Zhao Wang[2], Daniel Stoddard[1]

[1] Department of Biophysics, UT Southwestern Medical Center, Dallas, TX 75390, USA.
[2] Verna and Marrs McLean Department of Biochemistry and Molecular Pharmacology, Baylor College of Medicine, Houston, TX 77030, USA.

UT Southwestern Healthcare Summer Internship Program

Background

Cryo-focused ion beam milling is used to thin frozen biological samples into lamellae that can be used for cryo-electron tomography. Lamella thickness is an important factor because it affects electron transmission, image contrast, tilt-series quality, and the amount of cellular material preserved for analysis [1]. Samples that are too thick can produce greater inelastic scattering and lower-quality images, while excessive thinning of the sample may remove structures that are important for future analysis. During sample preparation, the Thermo Fisher Aquilos 2+ instrument provides an in-line estimate of lamella thickness. Thickness can also be evaluated after imaging by measuring reconstructed tomograms and by using energy-filtered TEM. Energy-filtered measurements compare total electron intensity with zero-loss intensity to estimate relative specimen thickness based on inelastic scattering [2]. Automated tilt-series acquisition supports the collection of images needed for three-dimensional reconstruction [3]. Comparing these methods can help identify consistent measurement differences and improve calibration between milling-stage estimates and post-imaging thickness measurements

Project Objective

This project compared Aquilos in-line thickness estimates, energy-filter count ratios, and tomogram-derived thickness measurements. The goals were to evaluate agreement between methods, quantify measurement discrepancies, and assess whether count ratio could support lamella-thickness calibration.

Materials and Methods

Vitrified *E. coli* samples were kindly provided by the Zhao Lab at Baylor College of Medicine. A 3 µL aliquot of sample was applied to R2/2 Quantifoil grids, back-blotted for 6 seconds, and plunge-frozen in liquid ethane using a Leica EM GP2. Cryo-lamellae spanning a range of thicknesses were prepared by cryo-focused ion beam (cryo-FIB) milling on a Thermo Fisher Aquilos 2+ instrument. Automated milling was performed overnight using AutoTEM, followed by final manual polishing at an ion beam current of 8 pA. After milling, an in-line Aquilos thickness estimate was recorded for each lamella.
To evaluate the Aquilos measurements, each lamella was imaged at 300 kV on a Thermo Fisher Titan Krios G2i transmission electron microscope using SerialEM software. The microscope was equipped with a Gatan BioQuantum energy filter and a K3 direct electron detector operated in counting mode. For each lamella, a zero-loss peak (ZLP)-filtered image and an unfiltered image of the same region were collected. Average electron counts were measured from both images, and the unfiltered-to-filtered count ratio was calculated as an independent indicator of specimen thickness because thicker samples produce greater inelastic electron scattering. Multiple tilt series were then collected from each lamella and reconstructed into three-dimensional tomograms using RELION 5.0, Etomo/IMOD, and AreTomo. Thickness was measured directly from the reconstructed tomograms, with repeated measurements averaged to obtain a representative tomogram- derived thickness for each lamella. The unfiltered/filtered count ratio was plotted against the corresponding tomogram thickness to evaluate its potential as a thickness-calibration measurement. Tomogram-derived values were then compared with the Aquilos in-line estimates to determine the magnitude and consistency of disagreement between the two measurement methods across the range of lamella thicknesses examined.

About Me

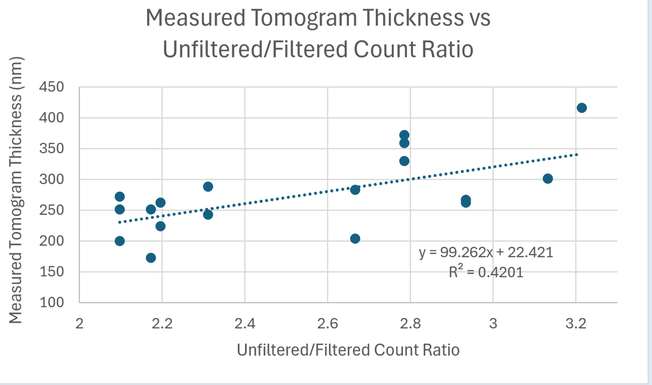
As a Biomedical Engineering student at The University of Texas at Austin, I am passionate about medical imaging, translational research, and medicine. I aspire to pursue a career in surgery alongside researching and developing advanced medical instrumentation. During my internship at the UT Southwestern Cryo-Electron Microscopy Facility, I gained hands-on experience in cryo-EM sample preparation, imaging workflows, quantitative data analysis, and scientific communication. This project provided an excellent opportunity to bridge engineering problem-solving with experimental biological imaging.

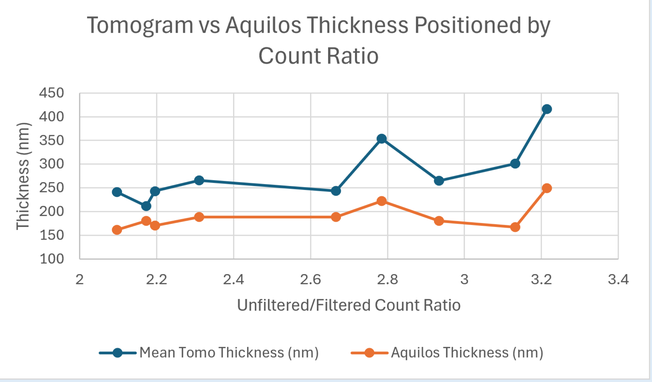
Penelope Paredes

Career Connection

This internship showed me how engineering, imaging, and biological research can work together to answer complex scientific questions.
The project strengthened my skills in quantitative image analysis, troubleshooting, experimental reasoning, and communicating technical results. These skills will be valuable as I continue studying biomedical engineering and pursue medical training.
The experience also reinforced my interest in applying engineering and research tools to improve medical imaging, clinical decision-making, and patient care.

Results





Top — Figure 1: Measured tomogram thickness is plotted against the unfiltered-to-filtered electron count ratio. Each point represents one tomogram, and multiple tomograms from the same lamella share the same count ratio. The dotted trendline summarizes the overall relationship between count ratio and thickness. A 151 nm measurement from Lamella4G5 was excluded as an outlier because this lamella showed substantial local thickness variation.

Bottom — Figure 2: Mean tomogram thickness and Aquilos thickness estimates are compared at each lamella's unfiltered-to-filtered count ratio. Each x-axis position represents one lamella, with repeated tomogram measurements averaged to obtain a representative thickness. Tomogram-derived values were consistently higher than the corresponding Aquilos estimates. Connecting lines are included only to aid visual comparison and do not represent continuous measurements between lamellae.

Key Results

Nine lamellae had matched energy-filter, tomo, and Aquilos measurements.

Mean tomo thickness: **282.2 nm**

Mean Aquilos thickness: **189.4 nm**

Mean difference: **+92.8 nm**

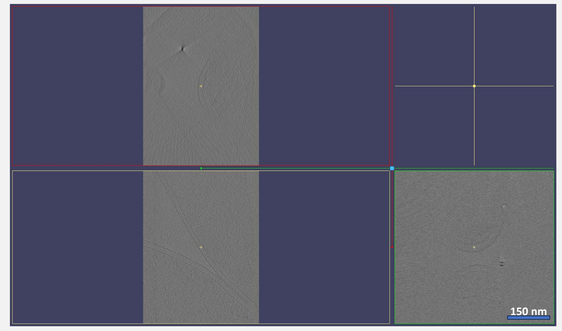
Tomogram measurements averaged 48.3% higher than Aquilos estimates.

Discussion

Tomogram-derived thickness measurements were consistently higher than the corresponding Aquilos estimates for the lamellae included in this dataset. On average, the tomo measurements were approximately 93 nm, or about 48%, higher than the Aquilos values. This suggests that the Aquilos in-line estimate may underestimate final lamella thickness under the conditions tested. However, the size of the discrepancy varied between lamellae, indicating that the difference between methods may not be explained by a single correction factor. The unfiltered-to-filtered count ratio generally increased with measured tomo thickness, suggesting that energy-filtered intensity measurements may provide useful information about relative lamella thickness. However, lamellae with similar count ratios sometimes had noticeably different tomo-derived thicknesses. This indicates that count ratio alone may be limited by the location within the lamella that images were taken.

Variation was also observed among tomograms collected from the same lamella. This suggests that thickness may not be uniform across the milled region and that a single measurement may not represent the entire lamella. Differences in measurement location, lamella geometry, reconstruction quality, edge effects, and local cellular or sample features may all contribute to the observed variability. These factors may also help explain why the Aquilos estimate and tomogram-derived measurements did not agree equally well for every lamella. Interpretation is limited by the small number of lamellae and by having only one filtered/unfiltered count pair for each lamella, even when multiple tomograms were measured. The energy-filter ratio therefore represents the lamella as a whole, while the tomo measurements may reflect different local regions. Future work should include more lamellae across a wider thickness range, collect energy-filter measurements at the exact tomogram locations, and evaluate variation across each lamella. These additional measurements would help determine whether a reliable calibration relationship can be developed among Aquilos estimates, energy-filter count ratios, and tomography-based thickness measurements.

Cryo-EM Workflow and Imaging



Top image — 3D Tomogram View
Orthogonal X, Y, and Z views of a reconstructed cryo-electron tomogram. Viewing the same volume from multiple planes illustrates the three-dimensional nature of the reconstruction and allows lamella thickness to be measured directly across the Z dimension.

Bottom left — Cryo-FIB Instrument
Thermo Fisher Aquilos 2+ cryo-focused ion beam instrument used to mill vitrified samples into thin cryo-lamellae and obtain the initial in-line thickness estimates.

Bottom center and right — Representative Tomograms
Looking in the Y and Z directions in a representative tomogram. These images were used to visualize the vitrified sample and directly measure lamella thickness for comparison with the Aquilos estimate and energy-filter count ratio. Scale bars represent 150 nm.

Conclusion

Tomogram-derived thickness measurements were consistently higher than Aquilos estimates, with an average difference of approximately 93 nm, or 48%. The unfiltered-to-filtered count ratio showed potential as an additional indicator of lamella thickness, although variation between lamellae and within individual lamellae remained substantial. These results suggest that Aquilos estimates, energy-filter measurements, and tomogram-derived thicknesses should not be treated as interchangeable without further calibration.

Next steps
- Collect more lamellae across a wider thickness range
- Match count measurements to the exact tomogram location
- Evaluate local thickness variation within each lamella
- Develop and validate a calibration relationship between methods

Challenges and Learning

One of the biggest challenges was learning an unfamiliar cryo-EM workflow and understanding how Aquilos estimates, tomogram measurements, and energy-filtered counts related to one another. The dataset also included repeated tomograms, missing measurements, thickness ranges, and a possible outlier, so I had to decide when to use individual values, averages, or exclusions. Another challenge was that only one filtered/unfiltered count pair was available for each lamella, even when several tomograms had been measured, which limited how directly the datasets could be matched. I also had to revise several graphs to make sure the x-axis, trendlines, and comparisons accurately represented the data.
Through this process, I improved my skills in Excel, data organization, statistical calculations, regression analysis, and scientific figure design. I learned to document assumptions, recognize limitations, respond to mentor feedback, and avoid overstating conclusions from a small dataset. Most importantly, I became more comfortable troubleshooting an open-ended research problem and communicating technical results clearly.

Acknowledgements and Sources

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Sources

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