Application note

Image data courtesy of Dr. Benjamin Engel, Dept. of Molecular Structural Biology, Max Planck Institute for Biochemistry, Martinsried, Germany.

Whole cell organelle segmentation with FIB-SEM and Amira Software

The activities of living cells are driven by a complex array of subcellular machinery. From mitochondria to microtubules, these intricately organized structures form a composite system capable of meeting any cellular demand. A complete understanding of organelle function depends on high-resolution 3D imaging of intact cells, which has long been elusive – until now. In this application note, we will explore a recent study from the Howard Hughes Medical Institute that combines focused ion beam scanning electron microscopy (FIB-SEM) whole-cell imaging, deep learning-based segmentation, and visualization using Thermo Scientific™ Amira™ Software to produce a comprehensive 3D map of the thousands of structures packed inside a single cell.[1]

Untangling subcellular structures

The functional basis of cells lies in the macromolecular assemblies and distinct membrane-bound structures known as organelles. Together, these molecular machines respire, transcribe DNA, fold proteins, and carry out the numerous other processes required for cells to live and function within organisms.

While the biochemical behavior and genetic nature of these subcellular structures are relatively well known, there is still much about them that remains a mystery. In particular, modern biology has long struggled to visualize and understand their 3D spatial distribution throughout cells, as well as their morphological complexities and interactions with each other across cells. The reason for this is simple: the only way to fully understand these behaviors is through 3D reconstruction of entire cells at nanometer-scale resolution. Even then, the immense size of the datasets, as well as the number and complexity of subcellular structures, require generalizable, automated methods.

Until recently, the main obstacles to this level of understanding were technological – there was simply no practical way of imaging subcellular structures at nanometer resolution over the comparatively large volume of an entire cell. Efforts to image cells in 3D using light microscopy allowed for some progress to be made, but the results were resolution-limited, and the introduction of fluorescent protein markers could introduce perturbations to the cellular system.

Many of these barriers have been overcome by the development of FIB-SEM, which combines the high-resolution imaging power of SEM with the precision milling of a FIB. Extremely thin layers of a sample are stripped away using FIB and sequentially imaged using SEM; these 2D slices can then be combined into 3D reconstructions of cells at extremely high resolution.

FIB-SEM was originally developed as a tool for semiconductor manufacturing and material research applications, which did not tend to require large-volume imaging. However, over the last decade, FIB-SEM has been repurposed as a powerful

tool for 3D biological imaging and used to produce high-resolution 3D images over relatively large volumes, such as entire eukaryotic cells.[2] Much of this progress has been driven by connectomics, the study of neuronal structures within organisms' nervous systems.

The primary advantage of FIB-SEM in these applications is that FIB affords extremely precise ablation of the surface, removing layers that are as thin as a few nanometers. This provides a much higher z-axis resolution compared to similar techniques such as diamond knife sectioning or block-face removal. FIB-SEM, therefore, affords highly "isotropic" 3D imaging, in which the resolution is approximately the same in the x, y and z directions – something that is essential for the imaging of complex 3D structures.[3,4]

Alongside developments in FIB-SEM hardware, significant innovations have been made in the automatic analysis of 3D electron microscopy data. Amira Software offers powerful visualization, segmentation, and processing of microscopy data (including FIB-SEM), providing biomedical and life-sciences researchers with the tools they need for whole cell chaacterization.

Methods

Researchers at the Howard Hughes Medical Institute, Virginia, developed a complete analytical pipeline for 3D reconstruction and analysis of organelles based on FIB-SEM imaging and Amira Software.

Cells from a number of different cell lines (i.e., HeLa, Jurkat, macrophage, and SUM159) were frozen at high pressure and embedded in resin. Layer by layer, each sample was then ablated and imaged by FIB-SEM in an automatic process.

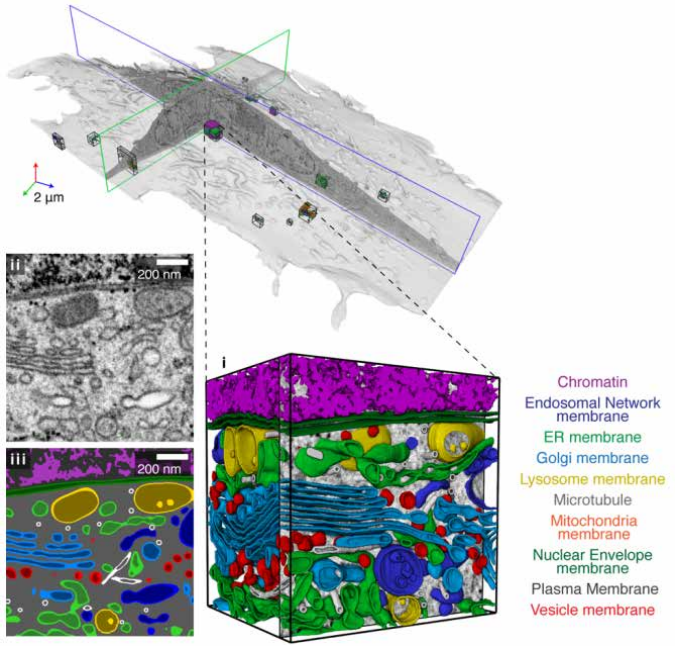

Figure 1. An example of a 1 µm³ "block" near the cell center, with manual annotations of subcellular structures.[1]

Visualizing data in Amira Software for algorithm training

This study primarily sought to develop a system for the automatic identification of subcellular structures using a machine-learning-based approach. In order to achieve this, "ground truth annotations" – *i.e.*, human expert classifications of structures – were required to train the algorithms.[5]

Amira Software was used to visualize data throughout the project. During the annotation/training phase, cropped and stacked TIF files – often 0.5 µm³ in volume – were imported into Amira Software for annotation. 3D transformation tools in the software were used to up-sample the data to 2 x 2 x 2 nm. Image clarity, contrast, and smoothness were then increased based on FIB-SEM staining variation. Regions of interest (referred to as "blocks") were then selected for manual annotation.

Expert annotators with backgrounds in biology and extensive experience viewing EM data used selection tools within Amira Software (such as brush, interpolation, fill, lasso, and smooth) to annotate and assign labels to all voxels. Up to 35 organelles and macromolecular structures were manually annotated on orthogonal XY, YZ, and XZ planes based on established morphological features. The resulting detailed, interactive 3D renderings were subsequently used as guides for further segmentation and organelle identification.

While structures such as mitochondria (with characteristic cristae) and long cylindrical microtubules were relatively straightforward to characterize, others were more challenging. The endoplasmic reticulum (ER), for example, can be identified by the way it connects back to itself and, ultimately, to the nuclear envelope. This means that complete volumetric datasets are typically required to identify the ER confidently.

The effort required to annotate the dense tangle of organelles in each block highlights the need for automation: manual segmentation of each organelle present per voxel within a single FIB-SEM slice took each expert about two weeks. At this rate, manual annotation of an entire cell would take one person around 60 years to complete.

The final training set consisted of 28 blocks from five datasets covering four different cell types, including 30 blocks that contained only extracellular space and 15 that only contained nucleus: up to 35 organelle classes were annotated in total. The "materials" feature of Amira Software was used to assign each organelle class a different "material" whose texture was smoothed, cleaned, properly identified, and separated from neighboring materials. Versatile data-handling features enabled blocks to be stored as chunked arrays in an N5 container, while metadata for each block was stored in a database.

Implementing the automated system

The manual annotations provided a robust basis for training the machine learning architectures to predict organelle segmentation from FIB-SEM data of whole resin-embedded cells.

While the automated segmentation of organelles was fast, the process was prolonged by the extremely high quantity of FIB-SEM images that are generated for a cell. Nonetheless, even using a single GPU, 14 organelle types could be inferred within an entire HeLa cell (a dataset consisting of 74 gigavoxels) in approximately 12.5 hours. (Note that regions containing exclusively resin were excluded to save valuable computation time.)

Evaluating performance

Raw predictions, smoothed with σ = 18 nm and rendered with cubic interpolation in Amira Software, showed organelle annotations that resembled those done manually. Indeed, the quality of these predictions immediately suggested that the networks could successfully reconstruct the organelles throughout the entire cell.

A manual evaluation method was developed to determine the optimal network for each organelle class. First, an organelle class was selected, and two corresponding 150 x 150 x 150 voxel crops were randomly selected from the volume, containing two different predictions of the selected organelle. By inspecting the crops (presented as image stacks) and selecting which prediction was more accurate (if either), researchers were able to test the relative performance of different predictions for each organelle within a data class.

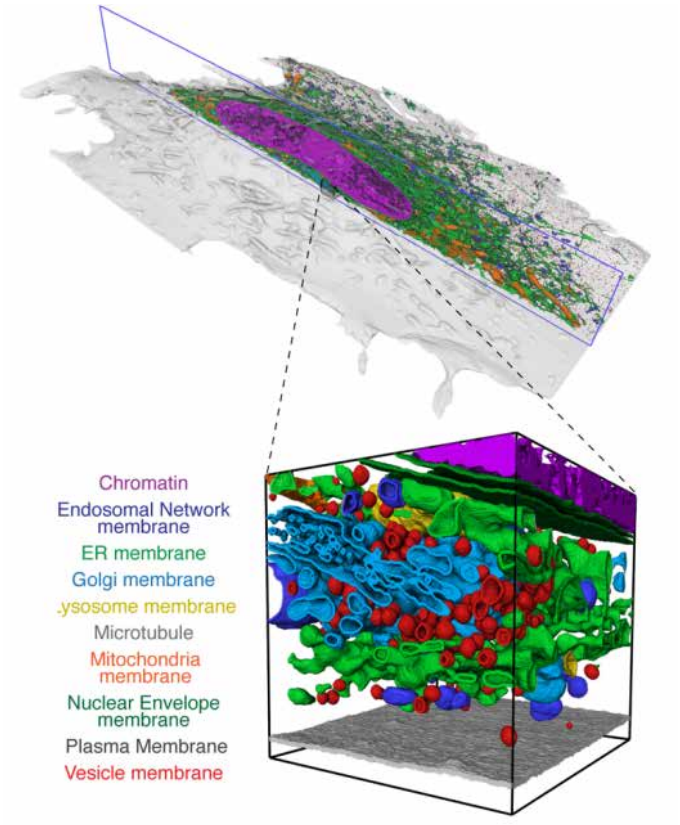

Figure 2. Automatic annotations by the trained networks, with quality similar to manual annotations.[1]

This method was used to test a range of 200,000 iterations for each network – enabling the optimal combination of network and iteration to be found.

In addition to this manual approach, a quantitative analysis of the methods was conducted by calculating the F1 score for each dataset as a performance measure. This enabled quantitative comparison between test and validation performance.
Overall, the results of the quantitative analysis showed that the automated system performed well for organelles that were well-represented in the training data, particularly for setups including many or all organelles.

Refinements

This statistical analysis allowed for some simple refinements that improved segmentation quality for certain organelles, including: smoothing, watershed segmentation and agglomeration, size filtering, and masking.[6] For other organelles, biological priors (i.e., microtubules) were used to improve predictions.

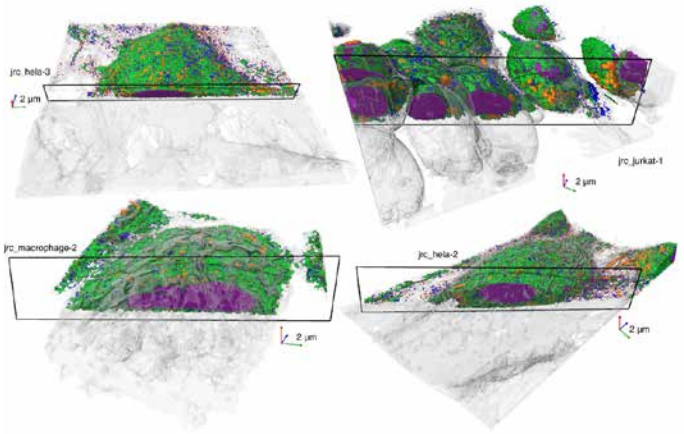

Figure 3. 3D renderings of refined organelle predictions for each dataset. Classes shown are plasma membrane (grey), ER (green), mitochondria (orange), nucleus (purple), endosomal system (blue), and vesicles (red).[1]

The resulting post-refinement whole-cell predictions are enormously complex. This complexity can be observed by focusing on the Golgi apparatus and associated vesicles within a small volume of a HeLa cell:

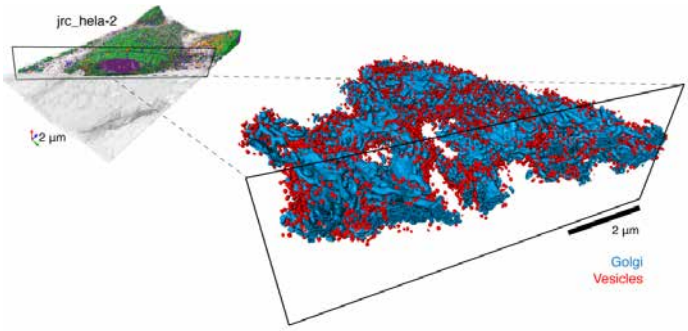

Figure 4. Amira visualization showing segmented Golgi apparatus and vesicles within a small volume of a HeLa cell.[1]

These 3D renderings highlight the true intricacy of cells compared with conventional textbook representations. The researchers note that, rather than being a compact stack of cisternae as is typically represented in textbooks, the Golgi apparatus appears to consist of flattened arrays of cisternae interconnected by narrow tubules, forming a winding ribbon that extends over 5 “µm” throughout the cytoplasm (approximately half the length of a typical animal cell).

Applications and analysis of data

Imaging, segmenting, and reconstructing whole cells at such a high level of detail provides access to a huge amount of information to elucidate cell structure. To illustrate this, the instance count, volume, and surface area of each organelle was calculated within the four different cells: a level of quantitative analysis that would be unimaginable using manual techniques.

These structural analyses can provide a biological basis for cell function: for example, a cell type involved in the destruction, phagocytosis, and detection of bacteria and other agents (the jrc_macrophage-2 cell) was found to have an increased volume ratio of ER, which could be relevant for its extensive membrane trafficking activity.

Additionally, the ability to “segment out” different organelles can be used to investigate the role of microtubules within cells. For example, counting organelles within 20 nm of a microtubule can quantify the number and type of organelles in contact with a single microtubule. These findings are unprecedented and open up new questions on the distribution and coordination of organelles along single microtubules.

The authors note that further work is required to generalize their reconstruction methods and make them robust across a range of cell types, tissues, and preparation methods. Nonetheless, this research represents a significant development in cell imaging and provides unique and valuable insight into organelle structure and organization in cells.

Conclusions

Amira Software is used throughout biomedical and life science research to provide cutting-edge visualization, processing, and analysis of microscopy data. Compatible with all major microscopy modalities, including optical and electron microscopy, (“µ”)CT, and MRI, Amira Software supports advanced 2D–5D bioimaging workflows with a suite of powerful and versatile tools.

Featuring powerful segmentation and automation capabilities, Amira Software can be customized and adapted into any workflow. Save time on complex analyses, produce publication-ready images and videos, and gain reliable answers to challenging questions.

References and further reading

1. Heinrich, L *et al. Automatic whole cell organelle segmentation in volumetric electron microscopy.* (Preprint) **bioRxiv** 2020.11.14.382143 (2020) NOTE: Later published as – Heinrich, L *et al. Whole-cell organelle segmentation in volume electron microscopy.* **Nature** 599 (2021)
2. Xu, CS et al. *Enhanced FIB-SEM systems for large-volume 3D imaging.* **eLife** 6 e25916 (2017)
3. Fischer, RS et al. *Microscopy in 3D: a biologist’s toolbox.* **Trends Cell Bio** 21 (2011)
4. Xu, CS et al. *Isotropic 3D electron microscopy reference library of whole cells and tissues.* **Nature** 599 (2021)
5. *The Solid Facts of Ground Truth Annotations* https://understand.ai/blog/annotation/machine-learning/autonomous-driving/2021/05/31/ground-truth-annotations.html
6. Zlateski, A and Seung, HS. *Image Segmentation by Size-Dependent Single Linkage Clustering of a Watershed Basin Graph.* **arXiv** 1505.00249 (2015)

Learn more at **thermofisher.com/amira**