Video Article

Electron Cryotomography of Bacterial Cells

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Abstract

While much is already known about the basic metabolism of bacterial cells, many fundamental questions are still surprisingly unanswered, including for instance how they generate and maintain specific cell shapes, establish polarity, segregate their genomes, and divide. In order to understand these phenomena, imaging technologies are needed that bridge the resolution gap between fluorescence light microscopy and higher-resolution methods such as X-ray crystallography and NMR spectroscopy.

Electron cryotomography (ECT) is an emerging technology that does just this, allowing the ultrastructure of cells to be visualized in a near-native state, in three dimensions (3D), with "macromolecular" resolution (~4nm).[1, 2] In ECT, cells are imaged in a vitreous, "frozen-hydrated" state in a cryo transmission electron microscope (cryoTEM) at low temperature (< -180°C). For slender cells (up to ~500 nm in thickness[3]), intact cells are plunge-frozen within media across EM grids in cryogens such as ethane or ethane/propane mixtures. Thicker cells and biofilms can also be imaged in a vitreous state by first "high-pressure freezing" and then, "cryo-sectioning" them. A series of two-dimensional projection images are then collected through the sample as it is incrementally tilted along one or two axes. A three-dimensional reconstruction, or "tomogram" can then be calculated from the images. While ECT requires expensive instrumentation, in recent years, it has been used in a few labs to reveal the structures of various external appendages, the structures of different cell envelopes, the positions and structures of cytoskeletal filaments, and the locations and architectures of large macromolecular assemblies such as flagellar motors, internal compartments and chemoreceptor arrays.[1, 2]

In this video article we illustrate how to image cells with ECT, including the processes of sample preparation, data collection, tomogram reconstruction, and interpretation of the results through segmentation and in some cases correlation with light microscopy.

Video Link

The video component of this article can be found at https://www.jove.com/video/1943/

Protocol

I. Preparation of Bacterial Cell Culture

1. Grow the bacterial cells in their normal media to desired phase in a few ml liquid culture. If higher concentration is needed, spinning and re-suspension in fresh media can be adopted, but be aware that this process sometimes introduces structural changes in the cells.
2. Check in the light microscope to make sure the culture is free of contaminants and at an appropriate confluence.
3. Some bacterial cells can stick or even grow on the EM grids. When growing cells on grids, gold grids with a carbon coat are most often used to avoid toxicity to the cells. They should be glow discharged for 2 minutes, sterilized under a UV light for 15 minutes, and placed directly into the liquid culture. Adherent cells will naturally stick to the grid, and others can be prompted to adhere to the grid by coating the grid in 0.1% poly-L-lysine first. Check the grids in the light microscope first to make sure the concentration of cells is appropriate before freezing.

II. Plunge freezing thin cells

For thin bacterial cells, the suspension of intact cells is plunge frozen across an EM grid. The process preserves the ultra-structure of the bacteria cells and will prevent water evaporation in the high vacuum of the EM later. It can be done either with custom-made plunge freezing devices, or as we show here, with a commercial automatic plunge freezer, FEI Vitrobot MKIII.[4]

1. Glow discharge the carbon coated EM grid for 2- 4 minutes. This makes the surface of the grid hydrophilic, thus helping to evenly spread the sample across the entire grid.

2. Mix 100µl colloidal gold solution (particle diameter of 10 nm) with 25µl of a 5% concentrated BSA solution. The BSA coats the gold particles and this prevents their aggregation.
3. Centrifuge the BSA and gold particle mixture at 18000g for 15 minutes. After removing the supernatant, the remaining gold pellet is mixed with 20 µl cell solution. The BSA treated gold particles do not react with the cells, but they will be needed as fiducial markers for reconstruction of the tomograms.
4. Apply 4 µl of the cell and gold solution mixture to the glow discharged EM grid in the Vitrobot at 100 % humidity. The grid is then automatically blotted from both sides with Nr 1 filter paper. This removes excess liquid, so that only a thin film of cells in their media remains. The grid is then plunged rapidly into a liquid mixture of ethane and propane that is cooled down to approximately 77K with liquid nitrogen. During this process, the sample is frozen so rapidly that the formation of ice crystals is prevented in thin films < 1 µm.
5. Carefully remove the grid from the ethane/propane mixture and quickly transfer into a grid storage box that is kept under liquid nitrogen.

III. High Pressure Freezing and Cryo Sectioning of Thicker Cells

For thicker cells, high pressure freezing reduces ice crystallization and subsequent cryo sectioning renders samples thin enough for EM imaging.[5, 6, 7] There are many different specimen holders available for high pressure freezing, your selection will depend on the sample, the type of freezing machine used or the investigative application selected. Here we use Bal-Tec HPM 010 high pressure freezer and cryo ultra microtome model UC6/FC6 from Leica Microsystems.

1. For bacteria, 15mls spinner culture OD >0.5 is taken directly from the 37°C incubator and centrifuged at 1000rpm for 3 minutes. After removing supernatant, mix 0.5ml of the remaining culture pellet with 0.5ml of 20% dextran cryoprotectant and then centrifuge at 13000rpm for 15 seconds.
2. After removing supernatant, pipette 0.1ml of the super pellet paste into heat-sealed micropipette tip which already contains 0.2ml 20% dextran cryoprotecant (40000Mwt). Centrifuge the mixture in the micropipette tip at 13000 rpm for 30 seconds and remove supernatant. A 5-10 µl tight pellet is seen at the sealed tip.
3. A "Teflon" coated brass-domed planchet ready mounted in the high pressure freezer holder arm receives the paste in one half-dome and is sealed with its flat partner brass hat.
4. The arm assembly is then promptly inserted into the primed high pressure freezer ready for freezing.
5. At 2100 bar pressure the combined brass planchet unit containing the cells is rapidly cooled by a jet of high pressure liquid nitrogen, removing the arm assembly instantly and plunging into a bath of liquid nitrogen prevents the planchet from warming.
6. Planchet units are stored under liquid nitrogen until required for cryo-sectioning.
7. To expose the well-frozen dome of cells for sectioning, the two brass planchets are carefully separated under liquid nitrogen.
8. The perfectly frozen dome of cells is glassy in appearance, free of cracks and ice nucleation fissures.
9. This planchet is transferred to a precooled -170°C chamber and mounted securely on the cryo-microtome vice-like chuck.
10. The cooled cryomicrotome contains essential cryotools necessary for sectioning, these include: a low angle cryo-diamond knife, a diamond trimming tool, press platform, grid storage box, an anti-static spray unit and a grid holding apparatus.
11. For successful ribbon sectioning an outer dome region is initially shaped to <0.2mm square by the diamond trimming tool and a rapid sectioning speed while retaining the ~200µm depth of well frozen perimeter.
12. With the antistatic spray directed at the low angle knife and copper support grids in close proximity, the diamond knife is carefully advanced to the polished block face readied for sectioning.
13. Microtome parameters are chosen, such as a section thickness setting between 50 to 350 nm, together with a cutting speed of ~0.4mm/sec and narrow cutting window. It is important to control the antistatic spray range and intensity to low humidity conditions in the immediate area.
14. As the first sections are produced a fine eyelash applicator is maneuvered by hand or micromanipulator to snag the early sections sliding off the low angle diamond knife edge.
15. While the ribbon of sections is forced off the knife, they are gently supported by an eyelash probe and guided across the copper grid support nearby.
16. To stick the sections, the grid loaded with ribbons is then firmly pressed using a cooled polished-silver probe, some sections are so highly charged the latest antistatic devices may assist in the adhesion process.
17. Grid boxes are kept for long term storage in a liquid nitrogen cooled dry shipper until the microscope is ready for grid transfer.

IV. Examine the Cells using the Cryo Light Microscope

The bacterial cells on the grid can be imaged using the cryo light microscope (cryoLM). The microscope we use here is a custom Nikon inverted microscope Ti E/B with extra long working distance (ELWD) 60x air objective lens. The grids are kept in a cryo stage during imaging, which is a modified FEI cryo stage for light microscope. Using a finder grid, the cells can be located on the grid squares and the cryoLM images be correlated to the cryoEM tomograms.

1. Load grids into cartridges on cryo-station. The cartridges are custom modified from our standard EM cartridge for the FEI TF30H-polara. The grid is held down by copper clip rings. The cartridge is then kept in a tube in liquid nitrogen for transferring.
2. Cool the cryo stage down to liquid nitrogen temperature (-190°C).
3. Load the cartridge into the cryo stage and move the grid into the viewing window. The stage can hold up to two custom modified cartridges.
4. Lower the condenser lens and focus the 60x objective lens onto grid.
5. Find the cells and take images. For correlated LM and EM study, the grid is scanned around the area of the cells, for locating the cells later in EM.
6. After imaging the grid, put the cartridge back into the tube and keep the tube in liquid nitrogen until ready to be imaged in EM.

V. Collect Tilt Series in the Cryo TEM

To obtain a 3D tomogram of the bacterial cell, a series of projection images are taken while the sample is incrementally tilted along one or two axes in the cryo TEM. Given the tilt angles and other experimental settings, there are several different software available to automatically collect the tilt series. We use Leginon for our cryo TEM which is FEI TF30H-polara. It allows for automatically taking tilt series of preselected cells.[8]

1. Load the cartridges into the multi-selection holder (MSH) on the precooled cryo station. The MSH can hold up to 6 cartridges.
2. Connect the MSH to the TF30H-polara, pick one cartridge and insert it into the EM column.
3. Start Leginon-client on the EM computer and Leginon main program on the other workstation.
4. Set up presets (image conditions) for a Leginon session. The important parameters are the magnification, the under focus value, the electron dose and the tilt angles for tilt series, including starting angle, end angle and increments.
5. Pick targets on images starting from the lowest magnification and send them to next step with higher magnification.
6. Queue up all targets for tilt-series collection and submit all of them to the final "tomography" step.
7. The process of data-collection can be monitored through the web-tool from a local workstation.
8. After the experimental data collection is done, download the completed tilt-series onto a local workstation for reconstruction.

VI. Biosafety considerations

Most of the biological samples that we work with are non-pathogenic and have been isolated from the soil, tap water or the gut of insects. Basic aseptic technique is sufficient for handling these samples. Working with pathogens requires the implementation of additional safety steps, however for extreme hazards, some labs have installed entire cryo-EM microscopes within BSL-3 labs. The following are some of the ways we have reduced the risk of working with pathogens in our lab.

1. The pathogenicity of a sample can be attenuated either chemically or genetically. Many labs doing cryo-EM of viruses have inactivated them with chemical fixatives prior to plunge-freezing. As an alternative, key mutations can be introduced that render the samples non-infective, including for instance inactivating point mutations in certain enzymes or knocking out receptors.
2. Personal protective gear such as gloves, lab coat and goggles should be worn.
3. Hazardous samples can be handled in a biosafety cabinet (BSC). Bacterial cultures can be grown, concentrated, and even plunge-frozen onto EM grids in the BSC. We have a smaller, manual plunge-freezing device that fits into our BSC for this purpose. If the greater consistency offered by automatic plunge-freezers is needed, they can either be introduced into the BSC, or, more simply, in certain cases we have backed the blotting pads of our Vitrobot with aluminum foil to prevent contamination of the device. When working with a BSC, as many of the tools and materials as possible going into or out of the cabinet should be disinfected with 70% ethanol. When preparing our grids, we use small volumes of a bacterial or viral culture (usually 4 ul), most of which gets blotted by the Whatman paper. The blotting paper, pipette tips, and remaining culture are disposed of as biohazardous waste. All tools and exposed surfaces are disinfected with diluted bleach, 95% ethanol and then water.
4. Once the grids are frozen, they are kept at liquid nitrogen temperatures throughout storage and data collection. It has been shown that cells can survive freezing and then thawing, though, so we continue to treat them as hazardous throughout storage and data collection. At the end of data collection, cartridges containing frozen grids are removed from the cryo-TEM and dropped directly into 70% alcohol. While rare, it should be noted that sometimes grids are "dropped" inside the column, and the consequences of such an event should be contemplated for each hazardous sample. Most of the microscope is at room temperature, so the sample on a dropped grid would likely thaw inside the column and become completely desiccated in the ultra-high vacuum of the EM. This would obviously inactivate some biological samples, but perhaps not robust viruses, and the desiccated remnants of the sample could potentially be released as aerosols when the microscope column is next opened for service.

VII. Tomogram Reconstruction

The tomograms of the bacterial cells are reconstructed through software. There are several software available for this job. We use Raptor for automatic reconstruction and screening tomograms.[9] As automated tilt series alignment and subsequent reconstruction is a non-trivial task, Raptor currently does not have a 100% success rate. In the case of Raptor failure or the need for a high-quality reconstruction, we use manual image alignment and reconstruction with eTomo in the IMOD tomography software suite.[10, 11]

1. Download each individual tilt series from the local Leginon webserver. Save them in a directory where they can easily be located.
2. Manually inspect the tilt series using IMOD's 3dmod viewer and discard those when Leginon has failed tracking the target or producing a useful tilt series.
3. We run Raptor distributed over the Linux machines in the lab through *Peach* (a Pearl-based system to distribute tasks over a network) . We specify the size of our fiducial markers, i.e. the colloidal gold particles, the number of markers to be used for reconstruction, the pixel-size, the desired thickness of reconstruction, the binning factor and a number of basic settings (such as input, output paths and output format) on the command line and leave it to run, generally 20min to 2h for a 122-image-dataset (2k by 2k). If satisfied with the reconstruction result, the steps below in this section can be skipped.
4. When manual reconstruction is needed, rename files from ".mrc" to ".st" and load individual tilt series into the eTomo GUI in the IMOD tomography suite.
5. Specify tilt series axis type (single or dual axes), and enter the fiducial size used in sample preparation. Scan the file header to determine pixel size, and proceed by clicking "Create Com scripts".
6. Follow the steps of each tab in the eTomo GUI to reconstruct the tomogram from the tilt series. Inspect and verify the result after each step, and if not satisfied, we can always go back to any previous step and reprocess from there. "Pre-processing" is to perform preparatory processing and remove abnormally high-intensity pixels caused by X-rays. "Coarse alignment" produces a tilt series wherein each subsequent projection image in the tilt series is aligned to the previous by cross-correlation. We define which gold beads to use as fiducials in "Fiducial Model Generation", and the program tracks the fiducial markers in each projection image and builds "Fine Alignment". "Tomogram

Positioning" allows cropping the tomogram in Z. "Final Aligned Stack" is generated using linear interpolation, and functions such as CTF-correction, Gold-Eraser and 2D-filtering can be applied optionally. The tomogram is calculated in "Generate Tomogram" by weighted back-projection in Fourier space. "Post-processing" enables cropping of the tomogram to the XY-region of interest and scaling the contrast in the range of the structures of interest, and "Clean Up" deletes the intermediate files and frees up the disk space.

VIII. Storage of the Tomograms in Database

We use a web-based in-house database to organize, store, search, and distribute the tomographic data. For each tilt series, we can record sample details, microscope collection parameters, raw tilt series as well as associated 3D reconstruction and other processing files. The main browsing page presents a thumbnail image and a featured "YouTube" like movie for each tomogram, and the entries can be organized by download frequency, creator, specimen type or date. Users can search the database by entering keywords or special features. Currently more than 4,500 tomographic tilt series have been deposited in the database, covering nearly 60 types of species/specimen, with a total of more than 11,000 associated files.

IX. Analysis and Segmentation of the Tomograms

1. The structural details of the tomograms can be viewed and analyzed through different software with image processing tools, e.g. 3dmod viewer of IMOD with ZAP, XYZ and Slicer window.
2. The next step is to create a segmentation of the 3D image (tomogram). A segmentation assigns to each voxel (volumetric pixel) of the 3D image a label describing to which region or material the voxel belongs, e. g., a membrane or a cytoskeleton. We use software tools such as 3dmod in IMOD and Amira (Visage Imaging).
3. Although some software tools provide automatic segmentation modules (e.g., threshold segmentation), they often only work for images with high contrast. The 3D images derived from low-dose electron cryotomography have such low contrast that in most cases we have to manually assign a label to each voxel.
4. The segmentation is stored in a separate data file. It can be used to generate a surface model in which each region is shown with a different color and viewed in 3D.
5. The tomograms can be used for other analysis. For example, template matching and subsequent calculation can yield statistical information of the macromolecular complex such as ribosomes. Subvolume alignment and averaging shows structures in higher contrast and reveals finer details.

X. Making movies based on tomograms

We make movies to summarize each project and to give a visual tour of our tomographic images.

1. Make a script of the features in the data we would like to show and how to render those features. For example, in a typical movie of a whole-cell tomography project, we start by showing a representative tilt series data and then follow with a slice-by-slice view of the reconstructed tomogram. Next, we show a segmentation of key macromolecules in the cell, for example, a flagellar motor or a chemoreceptor complex or a cytosolic filament. Finally, we conclude with a model showing an idealized interpretation of the macromolecules we are studying.
2. Graphics representation software such as Amira or Chimera are used to render the individual still frames of the movie. We typically assemble movies from shorter clips to facilitate changes to each clip.
3. Use commercial movie editing software such as Adobe Premiere to assemble the still frames into movie clips, and then the movie clips into the final movie.
4. We have started to add an audio track that narrates the movie. The narration acts as a "movie legend" and allows the audience to focus on the data being presented while watching the movie.

XI. Animation

Visual storytelling communicates knowledge without requiring the complex vocabulary of specific scientific domains. Difficult concepts become simple when accompanied by visual demonstration. The practice of illustrating biological ideas with cartoons is not new. However, only in the last decade have the sophisticated tools of professional 3D content creation been brought to bear on the task of constructing scientific animations. There are now dozens of researchers actively translating biological systems into dynamic 3D animations. Many beautiful animations are showcased on http://MolecularMovies.org. That site also hosts tutorials and distributes a free toolkit for manipulating molecular structures in Autodesk Maya. The open source 3D content suite, Blender, contains several user-generated PDB importers.

The process of making a 3D animation generally involves three steps:

1. Modeling: We use graphics representation software to export surfaces into formats that can be imported into Maya. These surfaces are either segmentations from reconstructed tomograms, for example, membranes, granules, or filaments, or representations of molecular surfaces. Tomogram segmentations are performed in Amira; molecular surfaces are generated in Chimera or VMD. Atomic representations of molecules can be imported directly into Maya from PDB files. Once the models are within Maya, they may be modified, duplicated, and positioned as appropriate.
2. Animating: Animations are traditionally constructed by hand, with the artist manually relocating moving objects and recording a series of key frames. New advances in 3D graphics software now allow dynamics to be generated procedurally, via built-in modules and scripting APIs.
3. Rendering: Procedural dynamics can provide convincing animations of dynamic biological processes, such as structural self-assembly. Yet due to the difficulty of realistically simulating biological systems, the majority of 3D animations are still built using traditional key frame techniques. Once the dynamics are in order, visual annotations, textures, and lighting effects may be added. Finally, the camera path is chosen and the completed animation is rendered into a movie.

Discussion

We show in this video article how to do ECT of bacterial cells. Limited by space and time, we only show the general protocol. More details can be found in papers, online or other information provided by the manufactures of the equipment and software developers, and from ECT research groups. Depending on the different bacterial species studied, the equipment used and the structural interest in the cells, the protocol and the experimental parameters need to be tested and optimized.

Disclosures

No conflicts of interest declared.

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References

1. Jensen, G.J. & Briegel, A. How electron cryotomography is opening a new window onto prokaryotic ultrastructure. Curr. Opin. Struct. Biol. 17, 260-267 (2007).
2. Li, Z. & Jensen, G.J. Electron cryotomography: a new view into microbial ultrastructure. Curr. Opin. Microbiol. 12, 333-340, (2009).
3. Frank, J. *et al.*, Three-dimensional imaging of biological complexity. J. Struct. Biol. 138, 85-91 (2002).
4. Iancu, C. V. *et al.*, Electron cryotomography sample preparation using the Vitrobot. Nat. Protocols 1, 2813-2819 (2007).
5. Studer, D., Graber, W., Al-Amoudi, A. & Eggli, P., A new approach for cryofixation by high-pressure freezing. J. Microsc. 203, 285-294 (2001).
6. Ladinsky, M.S., Pierson, J. & McIntosh, J.R., Vitreous cryosectioning of cells, facilitated by a micromanipulator. J. Microsc. 224, 129-134 (2006).
7. Pierson, J. *et al.*, Improving the technique of vitreous cryo-sectioning for cryo-electron tomography: Electrostatic charging for section attachment and implementation of an anti-contamination glove box. J. Struct. Biol. (2009), doi:10.1016/j.jsb.2009.10.001
8. Suloway, C. *et al.*, Fully automated, sequential tilt-series acquisition with Leginon. J. Struct. Biol. 167, 11-18 (2009).
9. Fernando, A. *et al.*, Markov random field based automatic image alignment for electron tomography. J. Struct. Biol. 161, 260-275 (2008).
10. Kremer, J.R., Mastronarde, D.N. & McIntosh, J.R., Computer visualization of three-dimensional image data using IMOD. J. Struct. Biol. 116, 71-76 (1996).
11. Mastronarde, D.N., Dual-axis tomography: an approach with alignment methods that preserve resolution. J. Struct. Biol. 120, 343-352 (1997).