TheScientist
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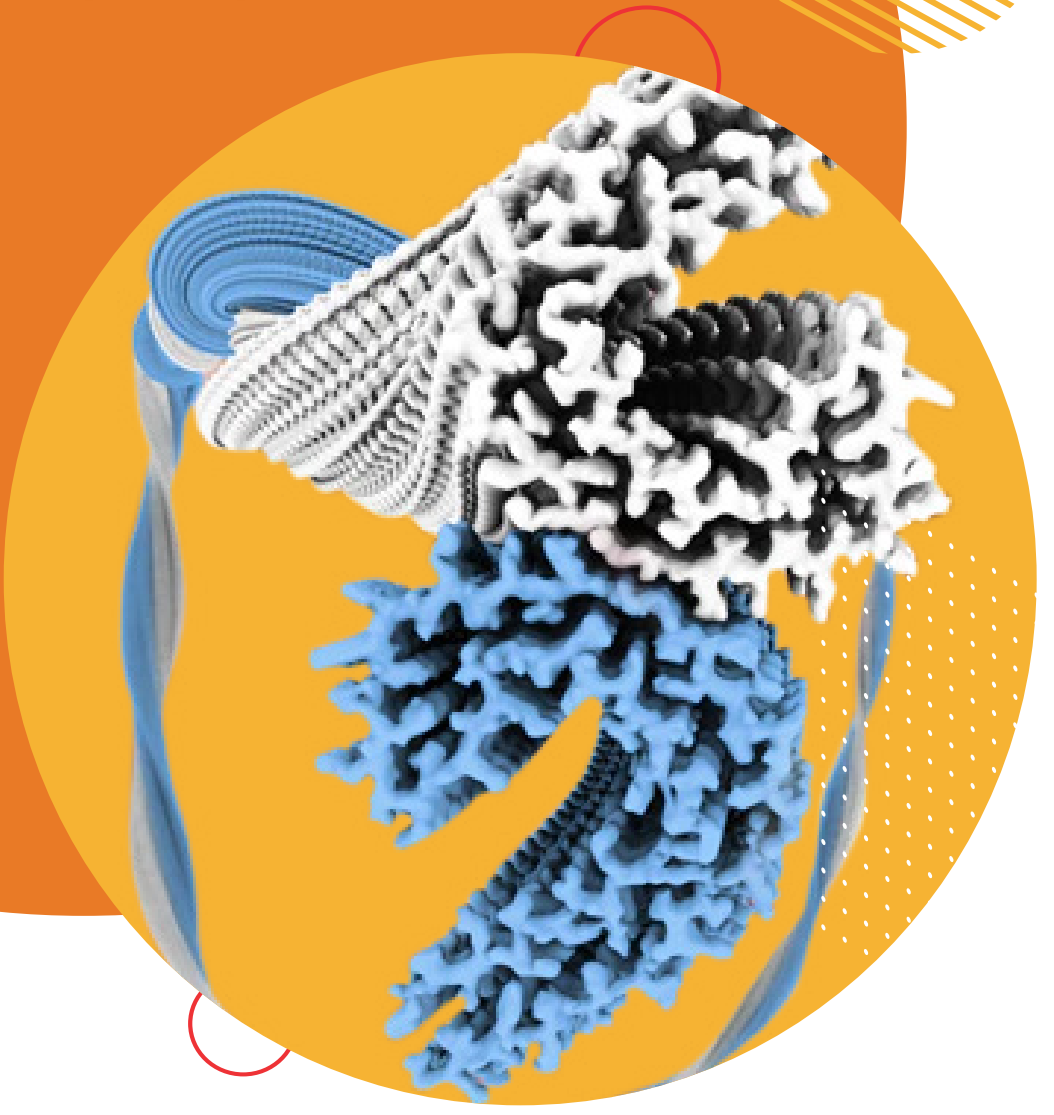
UNTANGLING NEURODEGENERATION WITH CRYO-EM



A CRYO-EM REVOLUTION FOR NEURODEGENERATIVE DISEASES

Neurodegenerative diseases are slow-progressing, incurable, and debilitating disorders affecting patients' physical movements and mental functions. Dementia is the most common symptom of neurodegeneration, with hallmarks of memory loss and reduced cognitive and language skills. According to the Global Brain Health Institute and the World Health Organization, the global cost of dementia is estimated to increase to $2 trillion by 2030, and the number of people with dementia is expected to triple to 150 million by 2050.[1,2] Due to the lack of prevention, a cure, or effective treatment, neurodegeneration is an imminent global health problem.

Protein Aggregates in Neurodegeneration

Alzheimer's, Parkinson's, and Huntington's diseases are common neurodegenerative disorders with accelerated neuronal death and loss of functional brain connections. Common histopathological hallmarks of these neurodegenerative diseases are long filaments and plaques that arise from disease-specific toxic proteins and form large protein aggregates in patient brains. For example, amyloid plaques and neurofibrillary tubulin-associated unit (tau) filaments are distinct structures in the brains of patients with Alzheimer's disease, where abnormal amyloid ß proteins accumulate in extracellular spaces and tau proteins cluster on neurons. In Huntington's patients, mutant huntingtin protein forms complex aggregates, and in Parkinson's patients α-synuclein aggregates clump in neurons to form Lewy bodies.

The link between protein aggregates and neurodegeneration remains unclear and controversial. In general, protein structure and function correlate. By understanding the structural features of these protein aggregates, scientists can address how they form, interact with the cellular environment, and alter brain function. Using structural biology tools, researchers begin to unravel the structural features of protein aggregates at atomic resolution to determine their pathology in brain tissue.

Structural Biology for Neurodegenerative Diseases

There are several structural biology techniques that resolve three-dimensional protein structures. Incorporating different methods such as x-ray crystallography, nuclear magnetic resonance (NMR), and cryogenic electron microscopy (cryo-EM), structural biologists map the atomic-level structures of molecules that are otherwise not visible with traditional high-resolution microscopy techniques.

Since its first use in 1953, x-ray crystallography has been a dominant method in structural biology research. Researchers purify and crystallize proteins of interest and use x-rays to generate an image of the protein molecules at atomic resolution. These structural images provide information such as structural interfaces, chemical bonds, binding sites, and protein-protein interactions. However, generating high-quality crystals is challenging, and complex protein aggregates in the form of filaments or tangles are impossible to crystallize. NMR spectroscopy offers an alternative to x-ray crystallography for proteins not amenable to crystallization. However, this method is limited to proteins with molecular weights below 50 kDa, so it is not compatible with high molecular weight protein aggregates like those found in neurodegenerative diseases. For example, researchers employed NMR spectroscopy to determine tau protein structure, but the method is not applicable for high molecular weight, complex tau tangles.

Recent developments in cryo-EM facilitate the profiling of large macromolecular proteins. Instead of crystallizing proteins, scientists performing cryo-EM flash-freeze (vitrify) protein solutions into a glass-like, hydrated state prior to imaging. Once in the electron microscope, a beam of accelerated electrons hits the protein sample. The protein molecules scatter the electrons, which produces images that show the atomic composition and arrangement of the protein.

With advancements in stable and multibeam microscopes to image larger sample areas, improved direct capture electron detectors, and faster software for image processing, cryo-EM is ideal for investigating the complex protein structures observed in neurodegenerative diseases. Such structural analyses are useful for understanding how proteins work, how they malfunction in disease, and how to target them with therapeutics. Researchers studying neurodegenerative diseases benefitted from the cryo-EM revolution and uncovered the atomic structures of numerous proteins that misfold in neurodegenerative diseases such as tau filaments, α-synuclein fibrils, and amyloid ß aggregates, as well as small molecule drug candidates that bind to these protein aggregates.[3]

See references on page 10.

CLASSIFYING TAUOPATHIES: DELINEATING ONE FILAMENT AT A TIME

Sjors H. W. Scheres, PhD
Medical Research Council Laboratory of Molecular Biology, Cambridge, UK

Tubulin-associated unit (tau) protein is essential for microtubule stabilization in neuronal filaments. The accumulation of abnormal tau proteins into filaments in the brain causes numerous neurodegenerative tauopathies. The tauopathies are clinically and pathologically separated into more than 20 diseases, with Alzheimer's disease being the most common.[1] Despite extensive clinical and neuropathological characterization, there is significant overlap between these disorders, which makes it difficult to obtain an accurate diagnosis. To solve this problem, structural biologist Sjors Scheres uses cryo-EM to distinguish new hallmarks of diseased tau filaments and classifies tauopathies based on their structures.[2]

Why is it important to decipher tau filament structures?

Even though this is just one protein from one gene, there is a family of diseases characterized by tau protein forming these filaments. A clinician can diagnose patients based on their symptoms or look at the post-mortem brain neuropathology from a light microscope to classify these diseases. This has been used for many years and it has been insightful.

That said, it is still difficult to distinguish some of these diseases. For example, progressive supranuclear palsy (PSP) was thought to be very similar to corticobasal degeneration (CBD) based on clinical diagnosis and neuropathology. We provide a third way to examine these diseases by solving the atomic structures of tau filaments that we extracted from postmortem brains. In addition, we have looked at many different diseases such as Alzheimer's disease, Pick's disease, and chronic traumatic encephalopathy. We discovered that each disease was characterized by different tau filament folds. Together, the differences between these folds form the basis of the structure-based classification. Although PSP and CBD are clinically and pathologically very similar, they are quite far apart based on the structures of filaments.

What are the advantages of using cryo-EM to study neurodegeneration?

We need to get atomic resolution information, and light microscopy is not good enough for that. With nuclear magnetic resonance (NMR), one would need to label samples with isotopes to see them in the magnet. You can only use NMR with recombinant proteins, and it is impossible to use it for human-derived specimens. The other main technique to solve structures is x-ray crystallography, for which one would need to crystallize tau filaments, which you cannot do. Here, cryo-EM has filled a niche in the field. The advantage of cryo-EM is you need relatively little brain-derived material. We use a gram of brain tissue and extract filaments from it. Because the filaments are long and heterogeneous, we do our sample preparation relatively dirty, which is advantageous because one can see many features in the cryo-EM structures.

Do cryo-EM structures have direct use in clinical applications?

People use positron emission tomography (PET) imaging where you have a radioactive ligand binding to a target. When a patient ingests the ligand, it goes to the target and PET imaging shows where it is in the body. There are PET ligands for tau filaments already in the clinic. We added those PET ligands to the brain-derived filaments and analyzed the structure to see where the ligand binds. Combined with the atomic coordinates of the filaments, computer simulations, and knowing that these filaments are different in each disease, you can design PET ligands that can distinguish between diseases, for example PSP or CBD.

In terms of studying disease, knowing the commonalities and differences between patients is useful. For example, we found a new structure that wasn't observed for any of the patients from other diseases we studied. The patient was misdiagnosed with PSP and was suffering from a novel disease, which we named Limbic-predominant neuronal inclusion body 4R tauopathy (LNT).[2] You can define new diseases and find commonalities and differences. The structures themselves can be used for ligand design, or perhaps even to develop inhibitors. Ultimately, we would like to find cures for these diseases.

See references on page 10.

This interview has been edited for length and clarity.

To learn more, watch **this video** where Anthony Fitzpatrick illustrates the power of cryo-EM in revealing Alzheimer's disease.

This video is courtesy of the Zuckerman Institute at Columbia University.

UNDERSTANDING THE CHEMICAL NATURE OF PROTEIN AGGREGATES

David S. Eisenberg, PhD
University of California, Los Angeles

Understanding how tau fibrils and amyloid plaques arise in the brains of Alzheimer's patients illuminates the progression from plaque formation to neurodegeneration. David Eisenberg and his team used cryo-EM to capture the details of amyloid fibers and understand the placement of atoms and the forces that hold them together. His team also analyzed the interaction between the candidate drug epigallocatechin-3-gallate (EGCG) and Alzheimer's brain-derived tau fibrils and discovered how the compound dissociates complex fibrils.[1]

"The part of the aging process is the weakening of systems that maintain the protein homeostasis in our cells, and for older people, the pathogenic fibers persist, grow, and cause damage."

—David S. Eisenberg

How do amyloid fibrils form?

Two main forces hold these fibrils together in the brains of people with neurodegenerative conditions. The first force is from hydrogen bonds (H-bonds), which is long recognized as being important in forming protein complexes. In the case of amyloid fibers, the individual molecules are flattened down in two-dimensions like pancakes rather than in intricate, three-dimensional patterns. Each molecule executes a series of curves within a flat, two-dimensional layer. The layers are then stacked on one another through numerous H-bonds, at least two per amino acid residue. There are hundreds of H-bonds, and each one is relatively weak, but with hundreds per layer, it is very significant. That is the first force that holds these fibers together.

The second force is steric zippers. The H-bonded layers form ß-sheets that run the whole length of the fiber. The zippers form between these sheets with one sheet bonding to the next one. This is a side-by-side rather than an up-and-down force like the hydrogen bonds. Together, they make extremely stable structures and are difficult to separate and get rid of once they form. Both the numbers of H-bonds and zippers contribute to stability.

What can break these stable bonds?

It has been known for decades that there are small molecules that can somehow disassemble amyloid fibers. One such molecule is EGCG. My colleague Paul Seidler and I became interested to know whether it would disassemble purified amyloid fibers taken from the autopsy brains of Alzheimer's patients. We isolated fibers from Alzheimer's brains and from the brains of patients who had related diseases. We found that EGCG readily disassembled these fibers overnight. If you wait three hours, EGCG is bound to the fiber and hasn't yet broken it up. We trapped EGCG bound to fibers and maneuvered them onto a grid—a small piece of copper with hundreds of holes in it—to visualize them in the electron microscope. We found that the molecule EGCG wedges into the crevice between two strands of tau molecules that make up the fiber. You can think of the fibers having two threads that twist around one another and the EGCG wedges into this crevice. When the EGCG wedges in, it forces the two strands apart such that they begin to disassemble. EGCG bristles with chemical groups that can form H-bonds, robbing the fiber from the H-bonds that it was using to hold itself together.

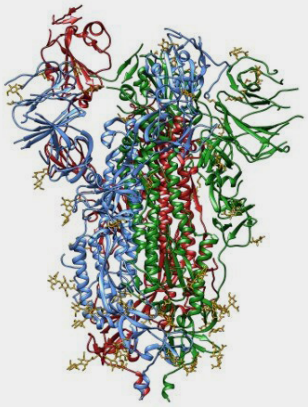
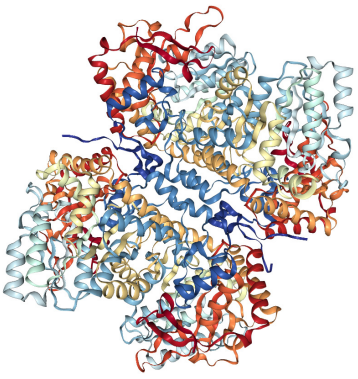
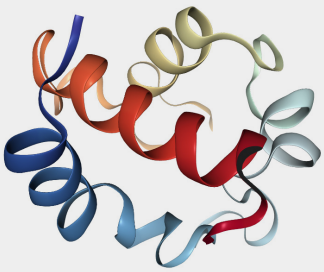
Is EGCG a suitable drug for neurodegenerative diseases?

EGCG is abundant in green tea and other foods, and it has been tried in several clinical trials, including for Alzheimer's and Parkinson's diseases. It has not been found to be effective because EGCG is reactive and gets modified in serum. It also cannot penetrate the blood-brain barrier. Molecules that easily get into the central nervous system tend to be non-polar, and EGCG is polar. EGCG also binds to many off-target proteins, making it unsuitable as a drug target. Even if EGCG is not a suitable drug, understanding its interaction with amyloid fibers at atomic resolution using cryo-EM can help discover or design other molecules that could become good drugs. My laboratory member, Kevin Murray, was able to find other small molecules that can also break down these fibers. We are pursuing them because maybe those could become useful drugs.

See references on page 10.

This interview has been edited for length and clarity.

Methods for Studying Protein Function

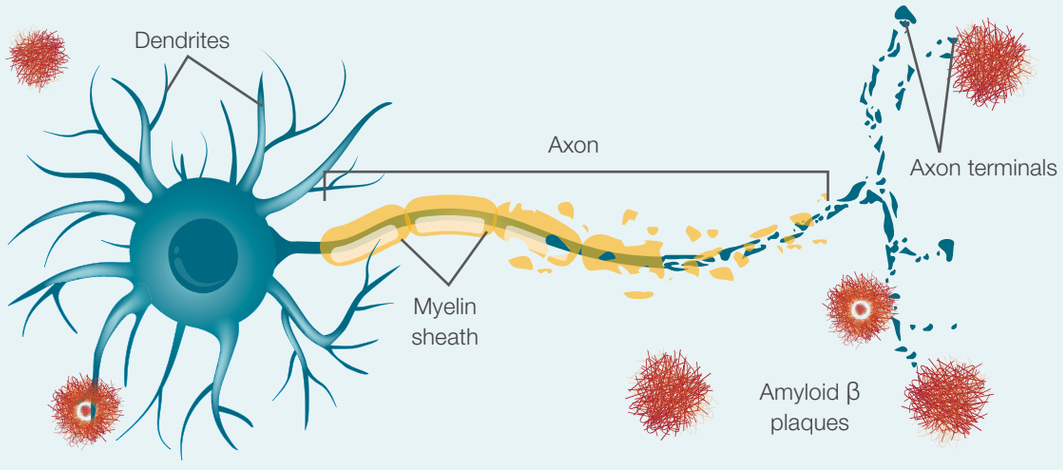
	 Cryo-EM	 X-ray crystallography	 NMR
Summary	Samples are rapidly frozen (vitrified), preserving the sample in its natural state. A transmission electron microscope (TEM) is used to capture two-dimensional projections of the specimen, which are then combined to make a 3D model.	Samples are crystalized and an X-ray beam is used to create a diffraction pattern from which the position of each atom in the crystallized molecule is determined.	Samples are subjected to a large magnet inside an NMR spectrometer. A series of split-second radio-wave pulses are applied to the sample, which forces the nuclei to resonate at specific frequencies. A complete picture of the protein is developed by combining the measured resonance frequencies.
Sample types	• Membrane proteins • Large complex proteins • Ribosomes • Virions • Other macromolecules	• Crystallizable samples • Soluble proteins	• Proteins with MWs <40–50 kDa
Advantages	• Easier sample preparation • Only requires small sample size • Structures are obtained in native state	• Works well for broad molecular weight ranges • Easier model building	• Obtains 3D structures in solution
Current limitations	• Proteins with molecular weights >100 kDa are preferred, but the number of structures from proteins with smaller molecular weights have increased as technology rapidly improves	• Crystallization can be difficult and can take months to years to achieve • Solid structure is preferred • Results in static crystalline state • Diffraction can be difficult	• Needs high purity sample • Has a difficult computational simulation • Sample must be isotopically labeled
Sample amounts required	Nanograms to micrograms	Micrograms to milligrams	Micrograms to milligrams

Solving the Amyloid Plaque Structure with Cryo-EM

Peeking into the Alzheimer's Brain

Extracellular amyloid β proteins form highly insoluble, densely-packed filaments in the brains of people with Alzheimer's disease. The proteins aggregate into long fibers forming a building block for plaques. The plaques accumulate between neurons and disrupt brain function, which contributes to the pathophysiology of Alzheimer's disease. Cryo-EM enables researchers to understand the structural features of amyloid plaques and how they form.

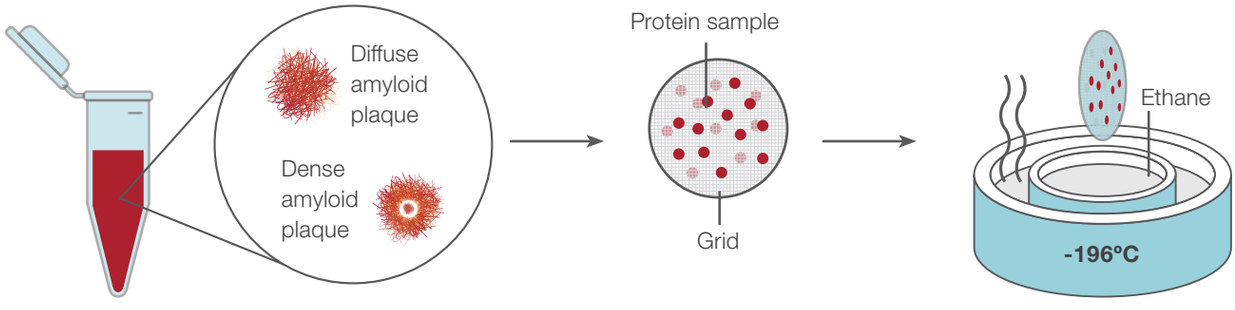
Plaque formation and neurodegeneration in Alzheimer's brain



Cryo-EM Sample Preparation Workflow

1. Purified amyloid filaments from the brains of Alzheimer's patients are transferred into a metal grid.

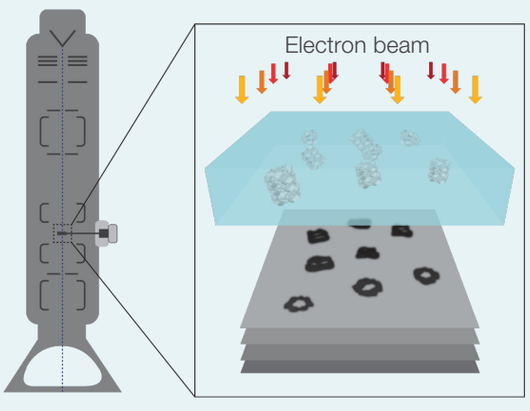
2. Fast-freezing the metal grid in ethane vitrifies water without forming ice crystals and readies the sample for measurements in the electron microscope.



The Anatomy of Cryo-EM and Image Processing

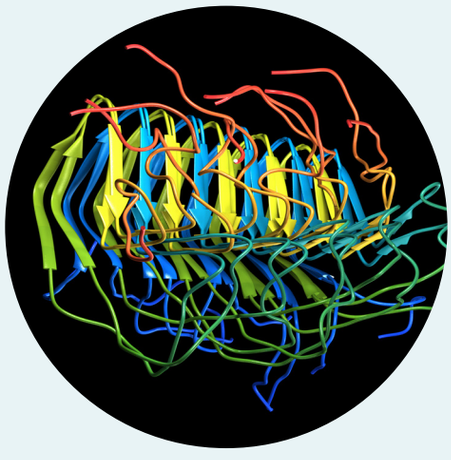
3. In cryo-EM imaging, an electron beam interacts with atoms in the vitrified protein sample. Different detectors register properties of scattered electrons producing high magnification images. Fast-freezing the metal grid in ethane vitrifies water without forming ice crystals and readies the sample for measurements in the electron microscope.

4. Powerful computational algorithms generate 3-D maps of amyloid proteins, reconstructed from thousands of high magnification 2-D images.



Vitrified amyloid filaments in the metal grid

High magnification 2-D images generated by the EM



SINGLE PARTICLE ANALYSIS WORKFLOW

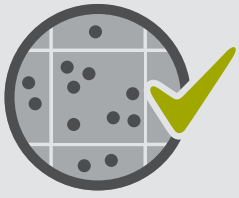
Single particle analysis is a revolutionary cryo-EM technique that has enabled the near-atomic structural determination of challenging proteins and protein complexes, without the need for crystallization. Samples can be studied directly in solution. High-quality data collection from cryo-EM has been facilitated by recent advances in sample preparation and data processing.

Sample preparation

High-quality cryo-EM starts with thorough sample preparation and screening. A variety of traditional sample preparation techniques can be used, including negative-stain screening and chromatography.

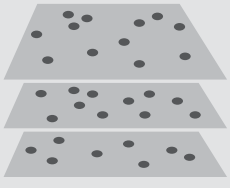
Vitrification

Once the aqueous sample is sufficiently purified, it must be rapidly frozen to suspend the specimens in a layer of amorphous (vitreous) ice (vitrification). By avoiding ice crystallization, the samples are preserved in a near-native state, essentially taking a snapshot of their structures in solution. Ice consistency as well as sample distribution and orientation are critical for data collection, and automated plunge freezing is the general method of choice for consistent sample vitrification.



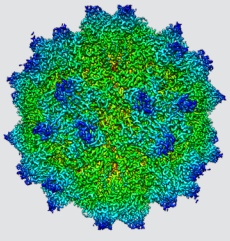
Screening

Even the best vitrification system is not 100% consistent, and therefore the sample (frozen atop an EM grid) must be screened to find the optimal areas of ice for data collection. Ideally, the ice would uniformly cover the holes in the grid, and there is a large amount of specimen distributed evenly throughout the visible ice. Only a moderate-resolution TEM scan is required at this stage, as this is a largely qualitative scan.



Data aquisition

Data collection consists of high-resolution imaging with a TEM specifically designed for cryo-applications (also called cryo-TEMs). With advances in data collection software, individual particles can be automatically identified in the TEM image and grouped according to particle orientation. For every sample, robust, reliable automation simplifies and accelerates imaging and identification.



Structure visualization

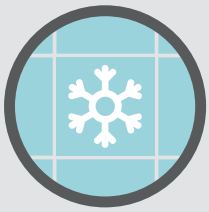
Once sufficient particle data is collected (ideally representing the sample from as many different orientations as possible) the data can be recombined into a 3D representation of the protein/protein complex. This uses 2D data from tens of thousands of particles and typically involves multiple data processing steps, requiring high data storage capacity and computational power. A number of professionally developed and open-source data processing solutions exist to simplify and expedite this process.

MicroED WORKFLOW

Microcrystal electron diffraction (MicroED) enables fast, high-resolution, structural determination of small molecules and proteins. Atomic details can be extracted from individual nanocrystals (<200 nm in size), even in a heterogeneous mixture. Data is acquired on a cryo-TEM, using electrons as the incident beam.

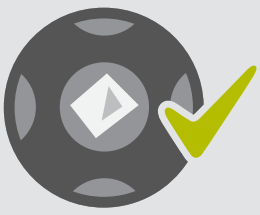
Sample preparation

The creation of small crystals for MicroED depends on their sample type. Small molecule crystals, which are usually dry, may require mechanical grinding, or they may simply be crystallized spontaneously out of solution using evaporation. Protein crystals are typically kept in water to retain their hydrated native states.



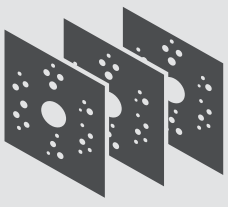
Vitrification

Protein crystals are prepared by plunge freezing, a method that is similar to the one used for single particle analysis. After freezing, crystals that are too thick for MicroED are thinned using a cryo-focused ion beam (cryo-FIB).



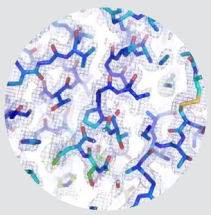
TEM low-dose screening

Continuous rotation data is collected from a single crystal within the electron beam on a fast camera.



Data collection

Individual images from the diffraction tilt series are combined computationally. Data collection is completed in only a few minutes, and 3D structures can be determined at atomic resolution.

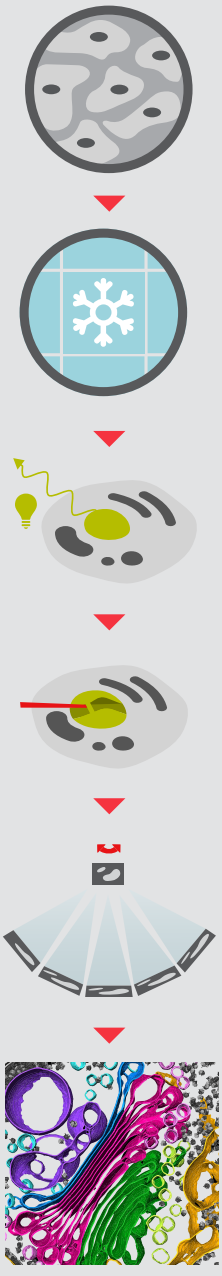


Reconstruction

The electron diffraction data obtained by this method is fully compatible with the available X-ray crystallography software, which simplifies analysis.

CRYO-ELECTRON TOMOGRAPHY WORKFLOW

Cryo-electron tomography (cryo-ET) provides label-free, fixation-free, nanometer-scale imaging of a cell's interior in 3D and visualizes protein complexes within their physiological environments. Using a correlative light and electron microscopy approach allows targeting of tagged proteins by fluorescence microscopy before subsequent cryo-EM higher-resolution imaging. Many cells are too thick for electrons, so the vitrified cells must be thinned with a cryo-focused ion beam microscope (cryo-FIB) prior to imaging in a transmission electron microscope.



Cell culture

Cells prepared by routine culture methods are grown on carbon-coated gold electron microscopy (EM) grids.

Sample preparation by vitrification

Cells are either vitrified through plunge-freezing (like SPA specimens) or High Pressure Freezing (HPF). The water in the sample freezes rapidly and does not crystallize, thus avoiding the molecular-scale disruption (by formed ice crystals) that would occur with a normal slow freezing process.

Localization by fluorescence

Using cryo-correlative microscopy the sample is transferred to a cryo-fluorescence light microscope (cryo-FLM), with which structures of interest are identified. A dedicated cryo-FLM stage keeps the sample in its vitrified state during cryo-fluorescence imaging.

Thinning by milling

A dedicated cryo-FIB prepares a thin, uniform lamella at the vitreous temperature (approximately -170°C).

Imaging by TEM

During cryo-ET, the sample is tilted in known increments about an axis. The individual projection images from the tomographic tilt series are then combined computationally in a procedure known as back-projection, which creates the 3D tomographic volume.

Reconstruction and visualization

The 3D tomogram featuring cellular structures can be segmented and colored in a variety of ways to enhance its display and presentation. From the tomogram small subsets of data containing the structures of interest can be computationally extracted and subjected to image processing methods.

References

Article 1: A Cryo-EM Revolution for Neurodegenerative Diseases

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2) "What is neurodegenerative disease," The EU Joint Programme – Neurodegenerative Disease Research, https://www.neurodegenerationresearch.eu/what/

3) H.A. Lashuel, "Rethinking protein aggregation and drug discovery in neurodegenerative diseases: Why we need to embrace complexity," *Curr Opin Chem Biol*, 64:67-75, 2021.

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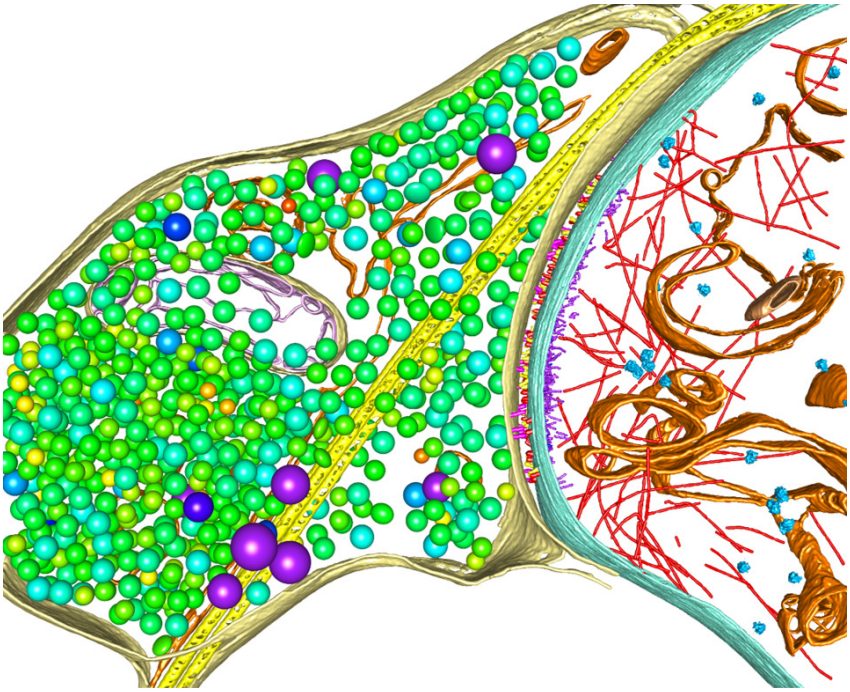
Article 2: Classifying Tauopathies: Delineating One Filament at a Time

1) R. Simões, I. Litvan, "Tauopathies," in *Encyclopedia of Movement Disorders*, Academic Press, 2010, pp. 219-26.

2) Y. Shi et al., "Structure-based classification of tauopathies," *Nature*, 598(7880):359-63, 2021.

Article 3: Understanding the Chemical Nature of Protein Aggregates

1) P.M. Seidler et al., "Cryo-EM reveals how the small molecule EGCG binds to Alzheimer's brain-derived tau fibrils and initiates fibril disaggregation," *bioRxiv*, 2021.



Ultrastructural organization underlying distinct synaptic functions

The image above shows a 3D view of an excitatory synapse between cultured hippocampal neurons revealed by cellular cryo-electron tomography. This cryo-EM method acquires 3D snapshots of the cellular interior and visualizes protein complexes within their crowded physiological environments. All essential structural synaptic elements are colored to facilitate visualization. Post-synaptic structures like PSD filaments and glutamate receptors are clearly depicted as well as pre-synaptic structures such as vesicles or adhesion proteins. Cryo-electron tomography gives a unique insight into the structure of synapses and will lead to a better understanding of the interplay of its components.

Tao C.-L., et al., "Differentiation and characterization of excitatory and inhibitory synapses by cryo-electron tomography and correlative microscopy, *JNeurosci*, 1548-17, 2018.

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Medium throughput	Dataset in 24 hours
Sample type	Proteins
Applications	SPA

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High-resolution SPA	200 kV, <2.5 Å*
High throughput	Dataset in 30 minutes
Sample type	Proteins, crystals, cells
Applications	SPA, MicroED, tomography

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- Our highest productivity and image quality cryo-TEM with an integrated workflow solution
- Our highest level of automation from sample vitrification to data analysis
- Compact design fits in standard room without costly renovations

Ultra-high-resolution SPA	300 kV, <1.5 Å*
Highest throughput	Dataset in minutes
Sample type	Proteins, crystals, cells
Applications	SPA, MicroED, tomography

* Based on best published performance, actual results will depend on non-microscope factors such as sample and user experience. Not a promise of biological resolution performance.

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

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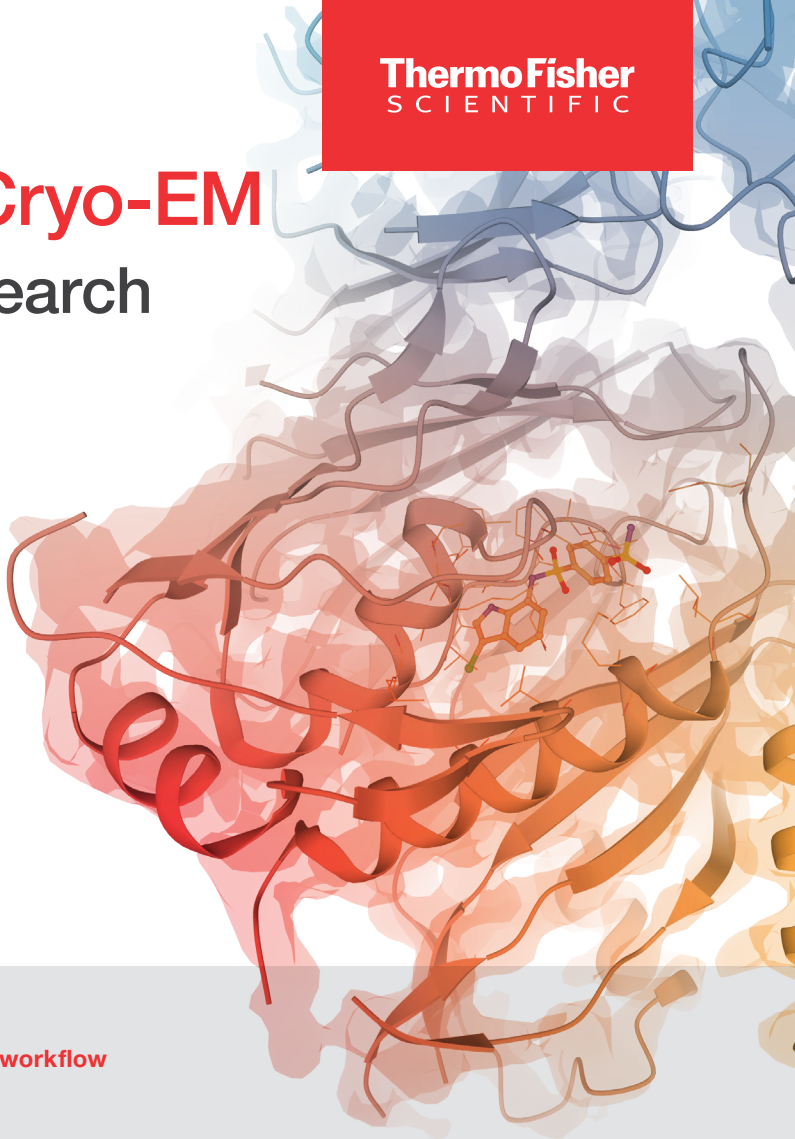
Getting Started in Cryo-EM

Revolutionize your research

Adopt cryo-EM quickly and seamlessly. As the market leader in cryo-EM, we can help you and your team be successful at every stage of the adoption process, from financing to guidance on facility and data-processing requirements. Find out how the Tundra and Glacios 2 Cryo-TEMs can provide entry-level solutions that fit your needs.



Products and support along every step of the cryo-EM workflow

Financing options
Competitive and flexible financing, leasing, lease-to-own options and more.

Site preparation services
Our experts provide guidance to minimize environmental interference and maximize system performance.

Installation
Post installation, we provide training for high-quality sample preparation and data collection using validated workflows.

Sample preparation for vitrification
Maximize sample quality with a range of products – from protein expression to purification and clean up.

Sample vitrification
Preserve biological integrity and quickly produce high-quality samples with the Thermo Scientific Vitrobot™ Mark IV System.

Data collection
The Tundra Cryo-TEM provides simplified single particle analysis, while the Glacios 2 Cryo-TEM offers improved efficiency, throughput, and ease of use for multiple applications.

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