**Structural Biology**
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**Lecture – 39**
**A concise story of advancement Cryo-EM**

Hi everyone, welcome again to the course on structural biology. We are continuing with the module on cryo-electron microscopy. In the previous three classes, I talked about microscopy, cryo-electron microscopy, different aspects of their sample preparation, instrument details, plunging, high-performance freezing, and data collection and analysis.

Today I want to summarise that in the form of a story, I hope you like it because cryo-electron microscopy is among the three high-resolution techniques and is considered to have the most potential one. How do the techniques travel? What are the challenges? What are the journeys? I am summarising them so you can see how it comes up.

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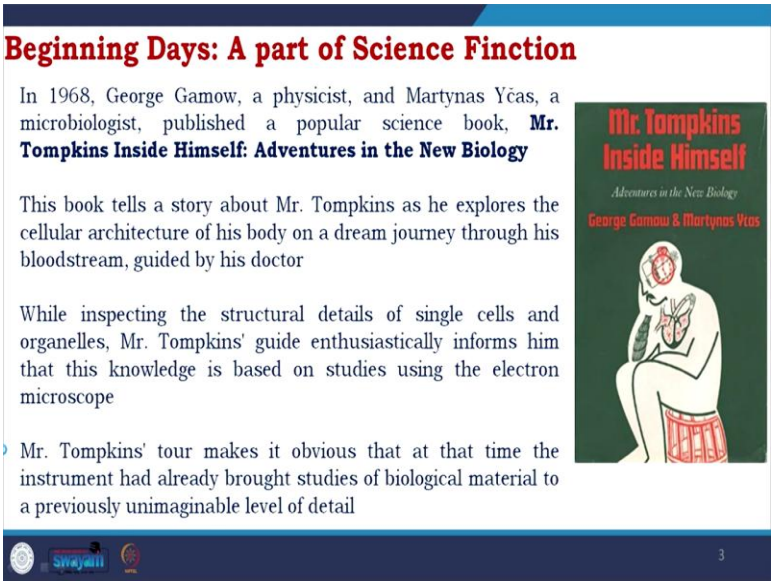


So concise story of the advancement of cryo-electron microscope, we will start with the beginning days, and there is a talk of a science fiction which happened in 1968 George Gamow, a physicist and Martynas Ycas, a microbiologist that published a popular science book called Mr Tompkins inside himself: The Adventures in the new Biology, what is this book about? This

book tells a story about Mr Tompkins as this guy explores the cellular architecture of his body on a dream journey through his bloodstream guided by his doctor.

It is a fantastic story to learn about our body and system how it works. More exciting things and more context to today's stories while inspecting the structural details of single cells and organelles Mr Tompkin's guide enthusiastically informs him that this knowledge is based on studies using the electron microscope. Mr Tompkins tour in the body makes it evident that this instrument already bought information from studies of biological material to a previously unimaginable level of detail. So, already people have started understanding the importance of electron microscopy.

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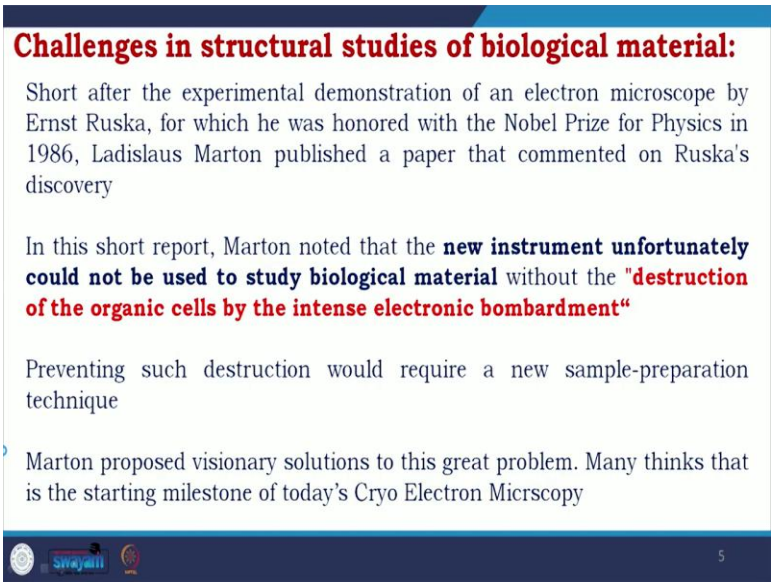


However, until just a few years ago, scientists could still dream of using the electron microscope to zoom in further into cells and organelles to uncover the atomic details of the biomolecules that underpin the architecture and the function. However, entry to the atomic level was impossible and challenging. This dream became a reality recently when a series of critical development made it possible to take full advantage of the pioneering discoveries and improvements made by the Nobel laureates Jacques Dubochet, Joachim Frank and Richard Henderson.

We will talk about them in detail while going to the stories. This advancement is now a structural determination of non-crystalline biomolecules in solution in high resolution using single-particle

cryo-electron microscopy. So this journey repeatedly talked about how important it is to get the three-dimensional structure of the biological macromolecules, especially protein.

Nevertheless, we have already discussed the challenges in crystallography. It is a very convenient technique gone through a long way, but it still needs the essential requirement to get a crystal, and the possibility of getting crystal for protein is low. Now, with the further advancement of the cryo-electron microscopy technique, you get the opportunity to use neo-native conditions to get the structure. Obviously, with the new knowledge of NMR, question what is happening in the NMR? NMR is a very potential instrument that we have to explore and much more than what is there, but it is challenging to get the protein in that high concentration that NMR demands in the current state.

Moreover, to get the structured way to go to the three-dimensional experiments; It is challenging to get that because it is expensive you have to get only the hydrogen isotope, which is NMR sensitive NMR active it is natural that all you have to provide, so that makes the cost of the getting the protein is high. In addition to that, in the presence of this unnatural isotope, protein also does not want to grow. So this is coming to continue with the study sought after the experimental demonstration of an electron microscope by Ernst Ruska, which he talked about for which he was honoured with Nobel Prize for Physics in 1986. Ladislaus Marton published a paper that commented on Ruska's discovery. Why is that paper vital? In a short report, Marton noted that the new instrument, the electron microscope, unfortunately, could not be used to study the biological material without the destruction of the organic cell by the intense electronic bombardments. So what he is trying to convey the message is that with the electronic bombardment, which is an integral part of the instrumentation working, it is not possible to make the biological materials intact it will destroy the material.

Preventing such destruction would require in new sample preparation technique. Marton is the first one who talks about that with alternative sample preparation; we could have started getting information about the biological sample using an electron microscope. Marton proposed an innovative solution to this significant problem, the starting milestone of today's cryo-electron microscopy solving the atomic structure.

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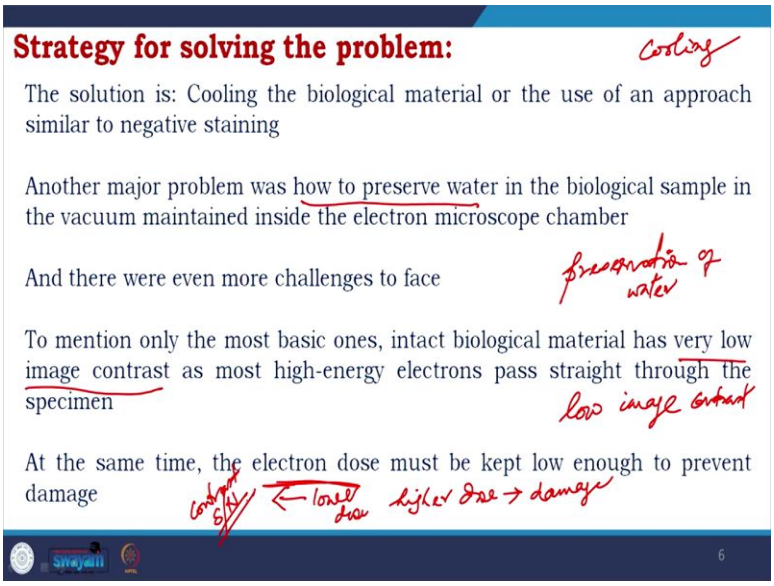


So, the strategy is to cool the biological material or use a negative staining approach. So Marton first talked about cooling, the key concept to cryo-electron. Another major problem was how to preserve the water in the biological sample in the vacuum maintained inside the electron microscope chamber. So you always want the protein to be in solution, so how to preserve the water? This is extremely important in the biological sample in the vacuum condition maintained inside the electron microscope chamber.

Moreover, there are even other challenges to face. The most basic one is that intact biological material has very low image contrast. Why? Because most high energy electrons pass straight to the specimen would not affect it, it has low image contrast. At the same time, the electron dose must be kept low enough to prevent damage. So, one preservation of water is a challenge.

A solution was cooling, and then the biological sample has low image contrast, and electron dose is important because higher dose damages now if you make lower dose contrast or signal by noise is even worse.

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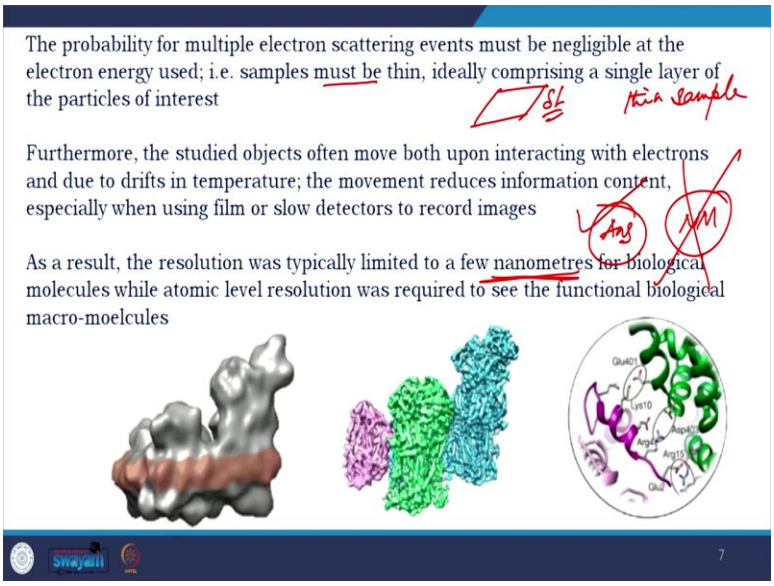


The probability for multiple electron scanning events must be negligible. If you see, when an electron hits a material like light hit material, there are many effects. Multiple electron scattering events must be negligible at the electron energy used to sample, so to do that, you have to have thin samples, ideally as a single layer. Furthermore, the studied objects often move both upon interacting with electrons, and due to drips in temperature, the movement reduces information content, especially when using film or slow detectors to record images.

This we have discussed in detail in the last portion. So I am not going into details you already understood at the time of data collection. As a result, the resolution was typically limited to a few nanometers for biological molecules. Well, the atomic-level resolution was required to see the functional biological macromolecules. So, we need it in angstroms, and this is what correct we get in nanometer, not correct.

So you see the earlier ones, but then it starts improving, and we are going literally to the higher resolution as you see here the atomic details that are the journey of cryo-electron microscope.

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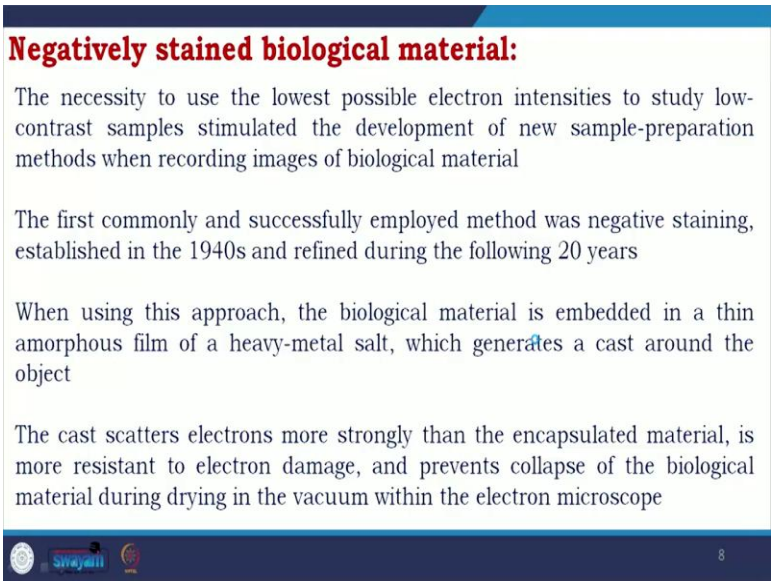


The negative staining of biological material plays a vital role even today's sample preparation checking the sample and initial development of the 2D model. Then necessity to use the lowest possible electron intensities to study low contrast samples stimulated the development of new sample preparation methods when recording images of biological materials. The first commonly and successfully employed method was negative staining established in 1940 and refined during the next 20 years with their alteration of the material.

How to use them? How to prepare them and all these things? When using this approach, the biological material is embedded in a thin amorphous film of a heavy metal salt which generates cost around the object. The cast scatters electron; we have already talked about this more strongly than the encapsulated material is more resistant to electron damage and prevents the biological material's collapse during drying in the vacuum within the electron microscope.

So the cast scatters electrons more strongly than the encapsulated material. So you have the encapsulated material and cast. The cast is more resistant to electron damage. So it is not directly coming to the biological sample and prevents the biological material's collapse during the drying in the vacuum within the electron microscope.

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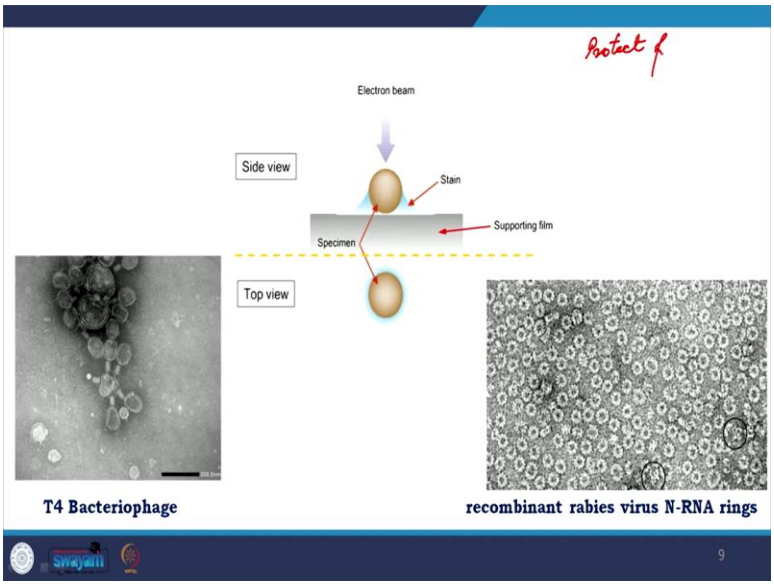


You could see the side view could see the top view. The electron beam is the same as protecting, made of the support film. This is negative staining of T4 bacteriophage. This is a negative side of the recombinant rabies virus in RNA rings. So by doing negative staining, you start looking at the thing you start getting better resolution because one it protects from damage and scatters more strongly, in addition, protect and protect from high vacuum setup.

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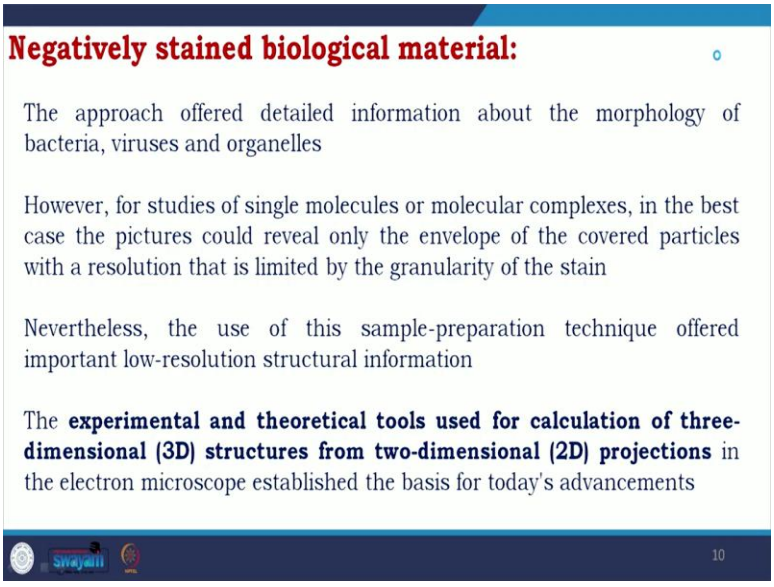


The approach of negative staining of detailed information about the morphology of bacteria, viruses, organelles as we have shown just. However, for studies of single molecules or molecular complexes, in the best case, the pictures could reveal all the; envelope of the covered particle

with a resolution limited by the granularity of the screen. So we are still unable to break the barrier of atomic resolution.

Nevertheless, using the sample preparation technique is crucial low-resolution structure information that, as I told for development of basic 2D material 2D model and all to check the homogeneity of the solution and all this is very good. The experimental and theoretical tool used to calculate three-dimensional structure from the two-dimensional projection in the electron microscope established the basis of today's advancement.

So, negative staining develops the experimental and theoretical tools used for calculating a three-dimensional structure from two-dimensional structures. We talk about this we talk about how it 2D picture different 2D pictures average and then using the tiltration using those changes we have developed 3D material.

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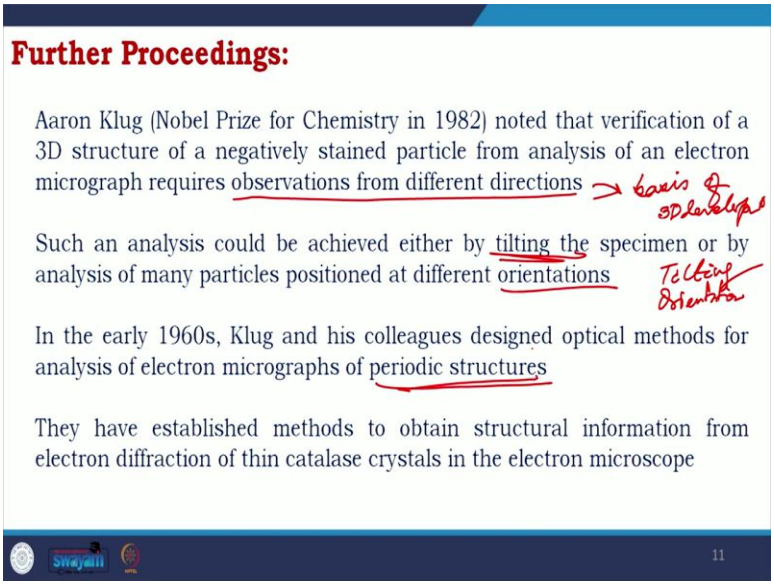


Aaron Klug was awarded the Nobel award of 1982 noted that verifying a 3D structure of a negatively stained particle from analysis of electron micrograph required observation from different directions. This analysis could be achieved by tilting the specimen; tilting is an important part of cryo-electron microscopy or analyzing many-particle positions at different orientations.

So one thing is tilting, another thing is orientation. We have talked about this earlier too. In early 1960, Klug and his colleagues designed optical methods to analyze the periodic structure's electron micrographs. So from the periodic structure, you could have developed models. There are established methods to obtain structural information from electron diffraction of thin catalyst enzymes in the electron microscope.

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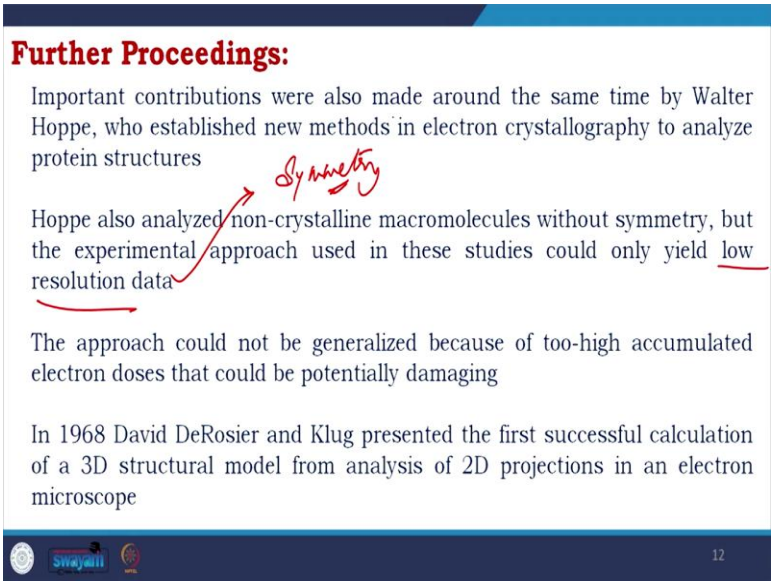


A vital contribution was also made around the same time by Walter Hope, who established a new method in electron crystallography to analyze the protein structure. Hope also analyzed the non-crystalline macromolecules without symmetry, but the experimental approach used in this study could yield low-resolution data telling the importance of symmetry. The process could not be generated because of too high accumulated electron doses that could potentially damage the sample.

In 1968 David DeRosier presented the first successful calculation of a 3D material model from an analysis of 2D projections in an electron microscope. So the first time that dream of converting a 2D picture to a 3D model is successful by DeRosier and Klug.

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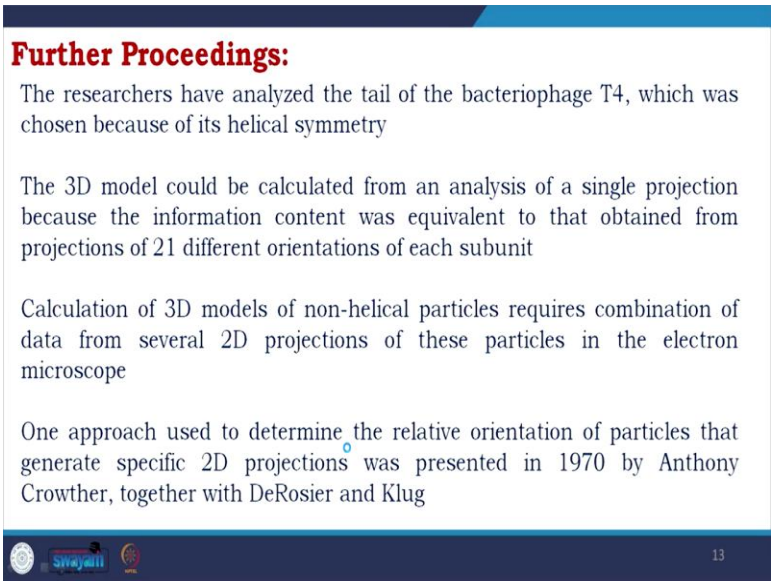


The researchers have analyzed the tail of the bacteriophage T4, which was chosen because of its helical symmetry. As I told symmetry is extremely important here. The 3D model could be calculated from an analyst with a single projection because the information content was equivalent to that obtained from the projection of 21 different orientations of each subunit. Calculation of 3D models of non-helical particles requires a combination of data from several 2D projections of these particles in the electron microscope.

One approach used to determine the relative orientation of the particles that generate specific 2D production was presented in 1970 by Anthony Crowther and the other people we talked about, Derosier and Klug.

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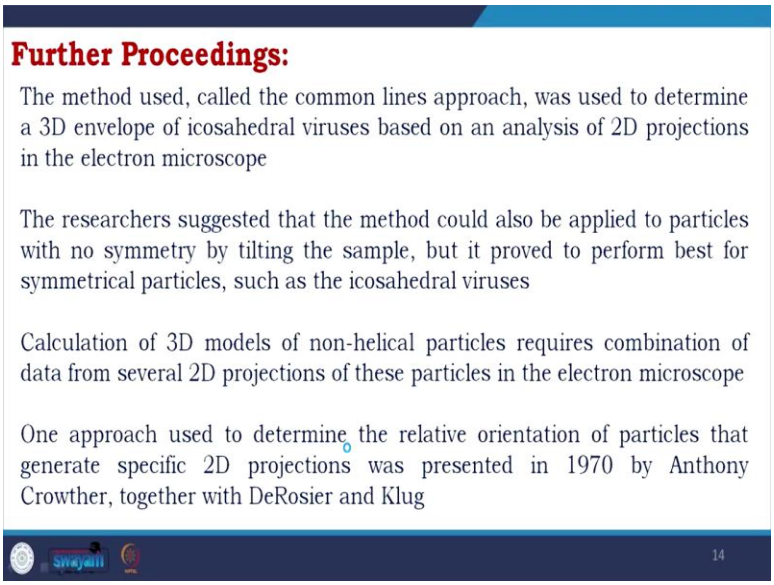


The method used to call the common lines approach was used to determine the 3D envelope of icosahedral viruses based on an analysis of 2D projection in the electron microscope. The researchers suggested that the method could also be applied to particles with no symmetry by tilting the temple, but its group performs best for symmetrical particles like the icosahedral virus, which maintains imaging symmetry in the structure.

Calculation of 3D models of non-helical particles requires a combination of data from several 2D projections. So, we have the micrograph pictures of the different 2D figures, get the projections and make the 3D model. One approach used to determine the relative orientation of particles that generate specific 2D projection was present in 1975 Anthony Crowther together with the DeRosier and Klug. The other people work continuously working on this.

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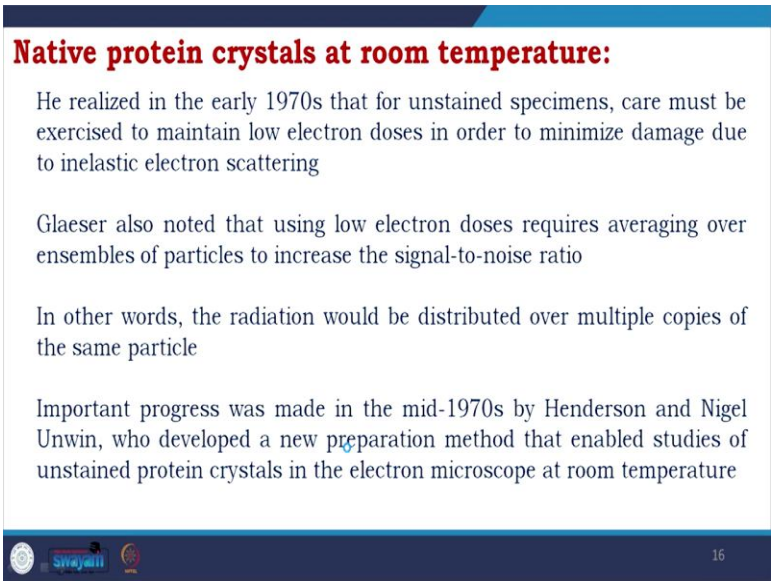


Now coming to; native protein crystal at room temperature: The techniques were also needed to preserve intact biomolecules in the hydrated state in the electron microscope and determine conditions for nondestructive irradiation. Donald Parsons developed environmental Chambers in which a humid atmosphere was maintained at room temperature in the electron microscope. If you remember, we talked about different such setups now, and you get them humid, or you get the auto control humidity, so that was the first time Donald Parson did that.

Using this approach is demonstrated electron diffraction from catalyst crystals and showed that it is possible to find conditions under which the protein structure is intact during the electron irradiation. Robert Glaeser quantified electrons induce radiation damage in studies of crystalline catalyst, and small organic molecules contributed significantly to further advancement of the field. He realized in early 1970 that for unstained specimens, care must be exercised to maintain low electron doses in order to minimize damage due to inelastic electron scattering.

Glacier also noted that using electron doses requires averaging over ensembles of particles to increase the signal to noise ratio. In other words, the radiation would be distributed over multiple copies of the same particle, and that would be good. Anderson made significant progress in mid-1970 Anderson and Nigel Unwin could develop a new preparation method that enables studies of unstrained protein crystal electron microscope at room temperature.

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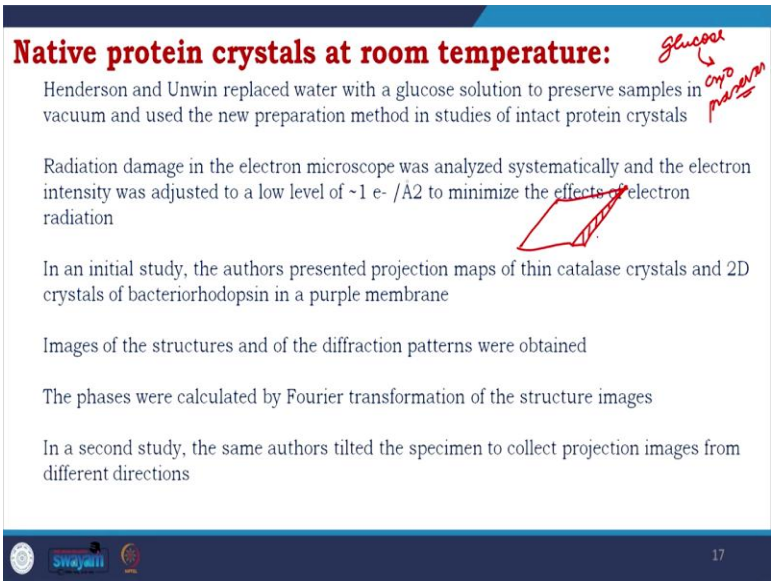


Henderson and Unwin replaced water with glucose solution, preserved the sample in a vacuum, and used the new preparation method to study an intact protein crystal. So kind of If you see when you are doing in crystallography, you use glucose solution as a cryo preserver. So Henderson, who started with initial studies as a crystallographer, got the idea there and used that. Radiation damage in the electron microscope was systematically analyzed, and electron intensity was adjusted at a low level to minimize the effect of the electron radiation.

In an initial study, the authors present projection maps of thin catalyst crystal and 2D crystals for bacteriorhodopsin in a purple membrane. So if you do not remember, 2D crystals are protein crystals that grow flat; I talked about them. So they do not have the dimension, and when you are talking about bacteriorhodopsin, you remember bacteriorhodopsin, the first protein Richard Henderson, coming up with the structure using electrons.

Images of the diffraction patterns were obtained. The phases were calculated by Fourier transformation of the structure images. In a second study, the same authors tilted the specimen as contributed by Klug to collect projection images from different directions.

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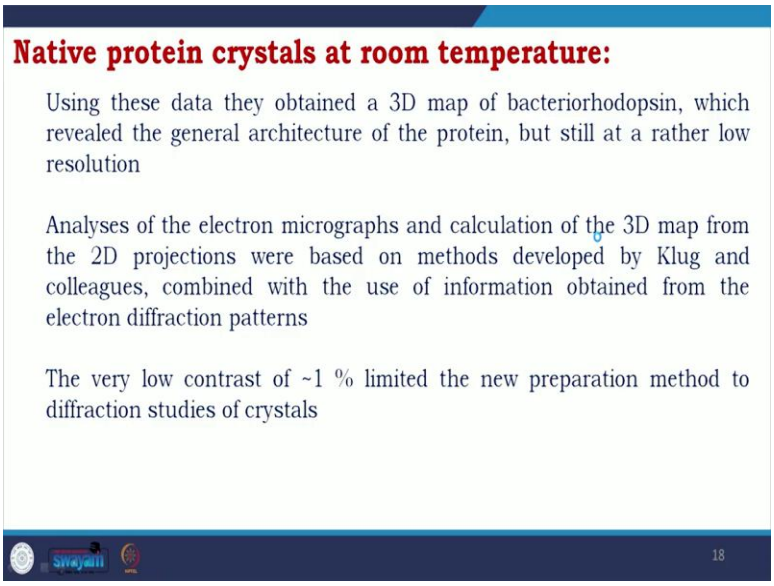


Using these data, they obtained the 3D map of bacteriorhodopsin, which revealed the general architecture of the protein but still at a relatively low resolution. Analysis of the electron micrographs and calculation of the 3D map from 2D projections are based on the method developed by Clug and Colleagues combined with the use of information obtained from the electron diffraction pattern. They used the tilting and all the processes Klug developed and added the electron diffraction pattern.

The very low contrast of one person 1% limited the new preparation method to diffraction studies of Crystal.

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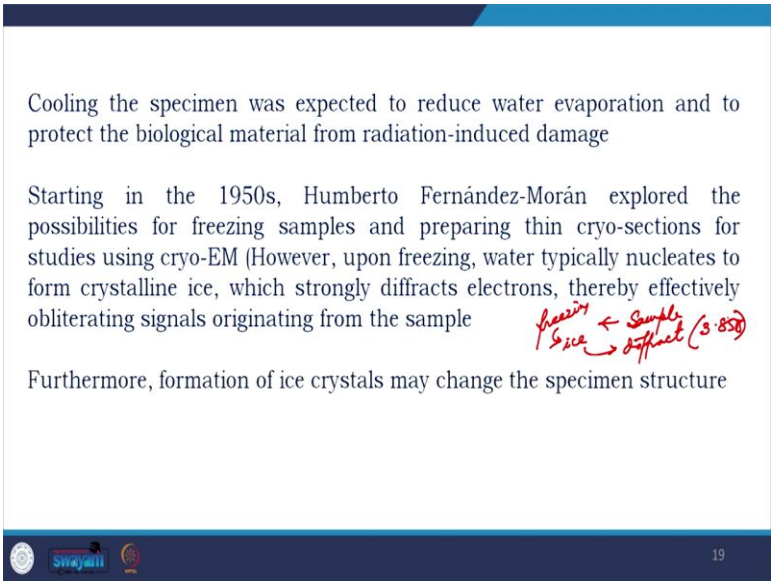

Cooling the specimen was expected to reduce water evaporation and protect the biological material from radiation-induced damage. Starting in 1950, Humberto Fernandez Moran explored the possibilities of freezing samples and preparing thin cryo-sections to study using cryo-EM. However, upon freezing, water typically nucleates to form crystalline ice that strongly defects electrons, thereby effectively making obliterating means making the process; the signal you do not get originated from the sample.

So what happened like you have the sample, and now you do the freezing. Freezing gives you ice crystals that diffract very strongly at around 4 angstroms. So, you get the signal coming from the water more than the signal coming from the sample. That was the problem. Furthermore, the formation of ice crystals may also change the structure of the specimen.

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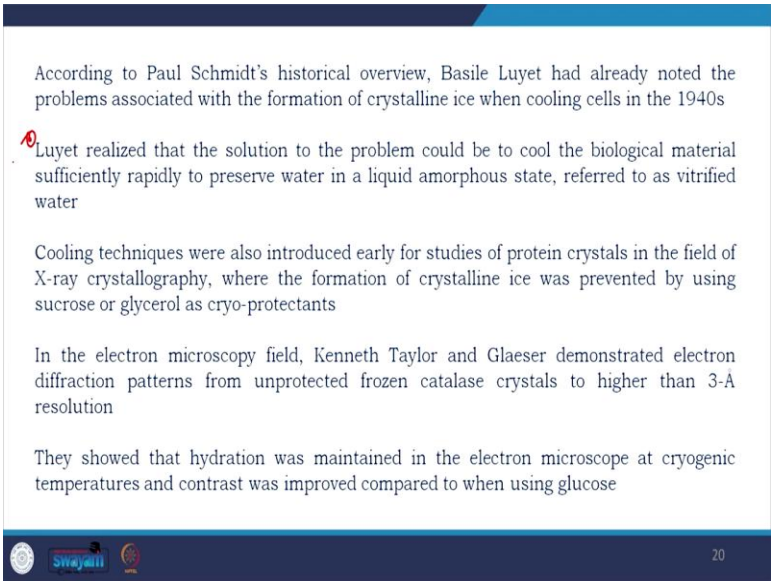


According to; Paul Schmidt historical overview, when you look back on the development of cryo-electron microscopy. Basil Luyet had already noted the problem associated with the formation of crystalline ice when cooling the cells in the 1940s. Luyet realized that the solution to the problem could be to cool the biological material sufficiently rapidly to preserve water in a liquid amorphous state, referred to as vitrified water.

So Luyet already has isolated the problem and understood that the development of vitrified water would be a good solution. Cooling techniques were also introduced early to study protein

crystals in X-Ray crystallography. So if you see that the cooling freezing techniques are very similar in protein crystallography and cryo-electron microscopy with the formation of crystalline ice was prevented by sucrose and glycerol as cryo-protectants, we still use that when you are freezing.

You need to freeze the crystal right when you are freezing; we use the cryo-protectant because, without the cryo-protectant, we will freeze the water, and water is a good diffractor. In the electron microscope field, Kenneth Taylor and Glaeser demonstrated an electron diffraction pattern from unprotected frozen catalyst crystal to higher than 3-angstrom resolution is a revolution. They showed that hydration was invented in the electron microscope at cryogenic temperature, and contrast was improved compared to when using glucose as a cryo-protectant.

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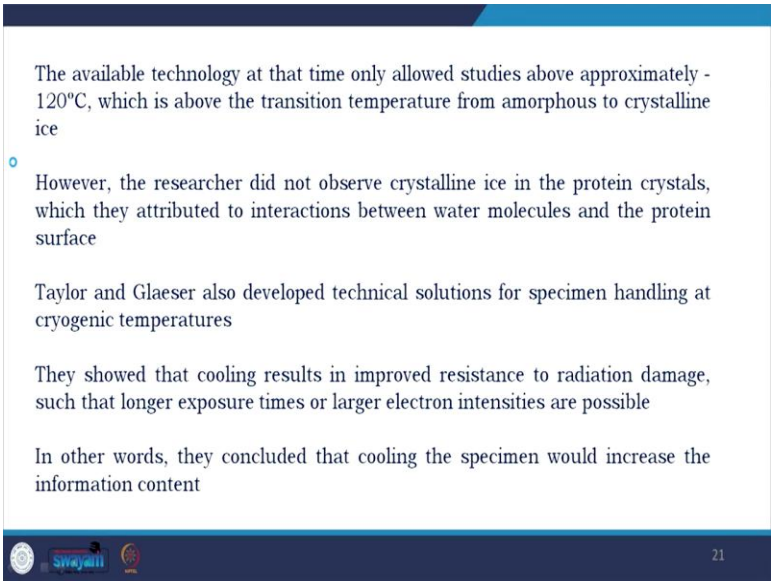


The available technology at the time only allowed studying from approximately minus 120 degrees centigrade, which is above the transition temperature from amorphous or crystalline. However, the researchers did not observe crystalline ice in the protein crystals, attributed to the interaction between the water molecule and the protein surface. Taylor and Glacier also developed a technical solution for specimen handling at cryogenic temperature.

They showed that cooling improves resistance to radiation damage such that longer exposure time and electron intensities are quite possible in this case. In other words, they concluded that cooling the specimen would increase the information content positively.

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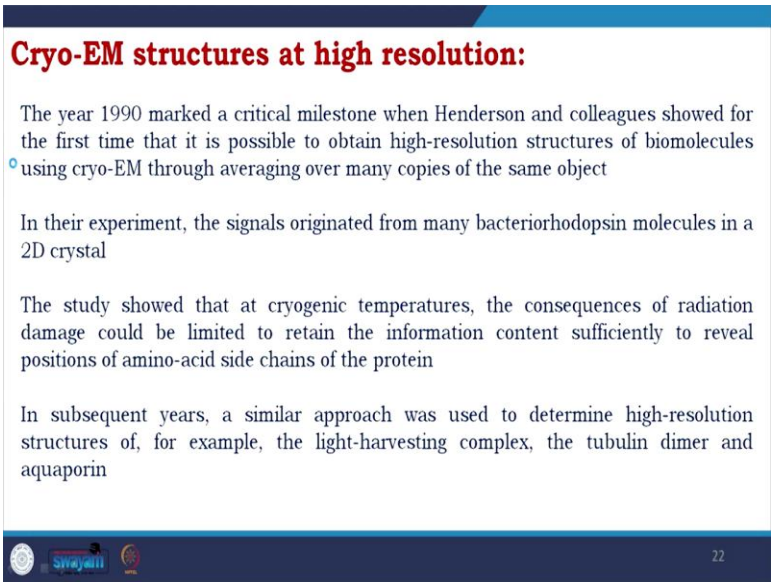


So this is the basic development, and now we will talk about cryo-EM. Today we have the cryo-EM structure at high resolution. The year 1990 marked a critical milestone when Henderson and colleagues showed for the first time that it is possible to obtain high-resolution structures for biomolecules using cryo-EM to average over many copies of the same object. Their experiment originated from many bacteriorhodopsin molecules in a 2D crystal.

The study showed that at cryogenic temperatures, the consequences of radiation damage could be limited to retaining the information content sufficiently to reveal the position of the amino acid side chain of the protein. In subsequent years a similar approach may be used to determine the high-resolution structure of the light-harvesting complex, the tubulin dimer and aquaporin, which are solved in high resolution.

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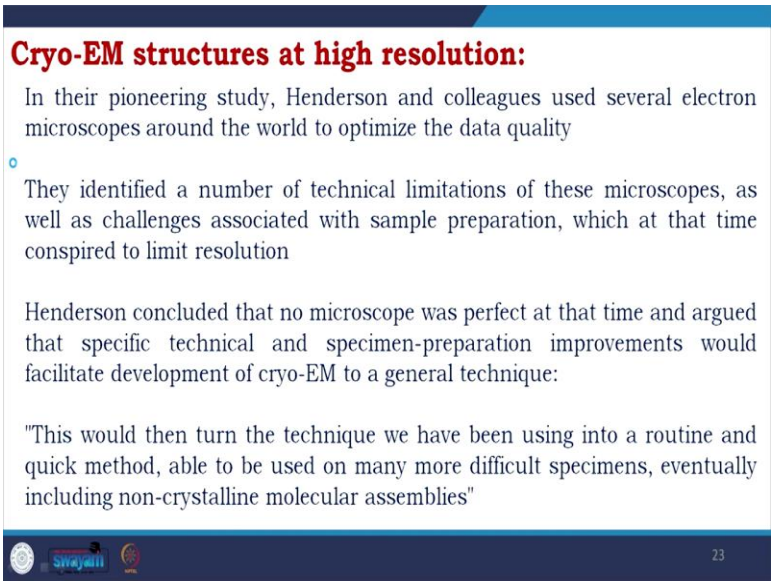


In their pioneering study, Henderson and colleagues used several electron microscopes worldwide to optimize the data quality. They identified the number of technical limitations of this microscope and challenges associated with sample preparation with advanced time conspires to limit resolution. So what do they do? They have one sample and travel around and access the different microscopes.

Moreover, try to make a comparison and find out the limiting factors. Henderson concluded that no microscope was perfect at the time and that specific technical and specimen preparation improvements would facilitate the development of cryo-EM to general technique; This would then turn the technique we have been using into a routine and quick method able to be used to much more difficult specimen eventually including non-crystalline molecular assemblies. So he was kind of a visionary in this case.

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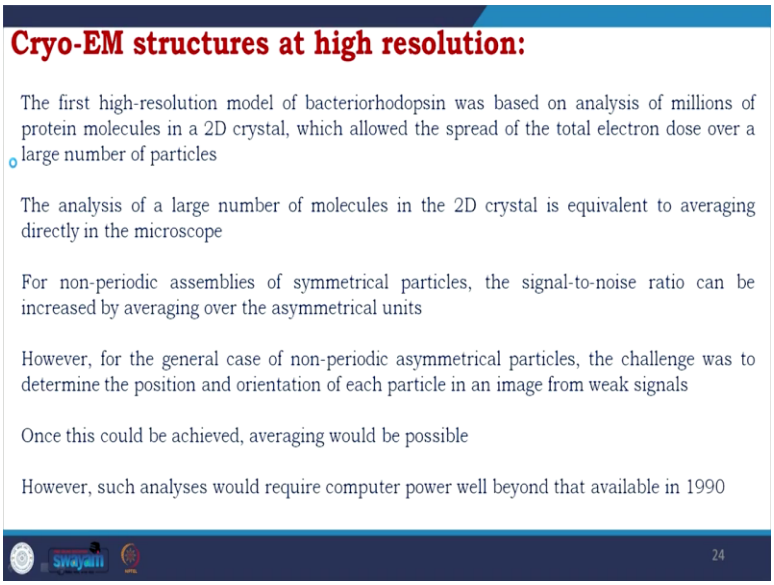


The first high-resolution model of bacteriorhodopsin was based on the analysis of millions of protein molecules in a 2D crystal which allowed the spread of the total electron dose over many particles. The analysis of many molecules in the 2D crystal is equivalent to averaging directly in the microscope. For non-periodic assemblies of symmetrical particles, the signal-to-noise ratio can be increased by averaging over the asymmetrical unit because the symmetry gives redundancy of the signal here.

However, in the general case of non-periodic asymmetrical particles, the challenge was to determine the position and orientation of each particle in an image from weak signals. Once this could be achieved, averaging is quite possible. However, such analysis would require computing power, which was available in 1990 because there are many micrographs you have to do a simulation.

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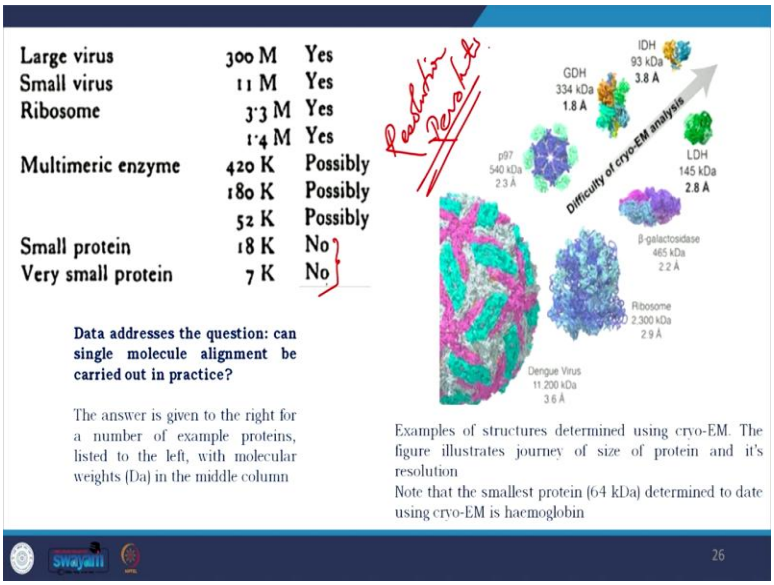


Five years after the solution of the high-resolution structure of bacteriorhodopsin, Henderson presented a quantitative analysis of challenges needed to overcome to determine the atomic resolution structure of non-crystalline molecular assemblies. We concluded by using low-intensity nondestructive electron irradiation in phase contrast electron microscopy. It would be possible to determine an individual particle's 2D position and sufficiently high molecular weight orientation.

The information would allow averaging of randomly distributed particles in ensembles, thereby eventually reaching an atomic resolution. So the first initiative is when we are on track. The conclusion for Henderson work was that assuming a molecular weight higher than 50, kilodalton it should be possible to align an average reasonable number 104, so 50 kilodalton here his condition was higher than 50 kilodaltons molecular weight.

Average with 104, a particle should determine the structure is possible at 3 angstroms. So, 50; kilodalton 104; particle 3-angstrom resolution. So here is a comparison for large viruses where the mass is 300 megadalton. It is possible for the small virus to 11Megadalton possible for ribosome 3.3 megadalton and 1.4 megadaltons, for Eukaryotic and prokaryotic possible multi-enzyme 420 kilodalton, 180 kilodaltons, 52 kilodaltons.

Yes, possible small protein or very small protein-peptide it is not. So this data addresses the question can single-molecule alignment be carried out in practice. So the answer is given to the right for the number of example proteins listed to the left with molecular weight in the middle colour. However, now you see here, I would talk about this, which is called the resolution in cryo-electron microscopy.

So, examples of structures determined using cryo-EM the figure elastic the size you will see that 11200 kilodaltons, dengue virus solved at 3.6 angstroms from P97 540 kilodalton solved at 2.3 angstroms, ribosome 2300 kilodalton solved at 2.9 angstroms, beta-galactosidase 465 kilodalton solvent 2.2 angstroms, GDH 334 kilodalton 1.8-angstrom LDH 145 kilodalton 2.8 angstroms and IDH 93 kilodalton 3.8 angstroms. Also, the smallest one is haemoglobin which is solved at 64 kilodalton size.

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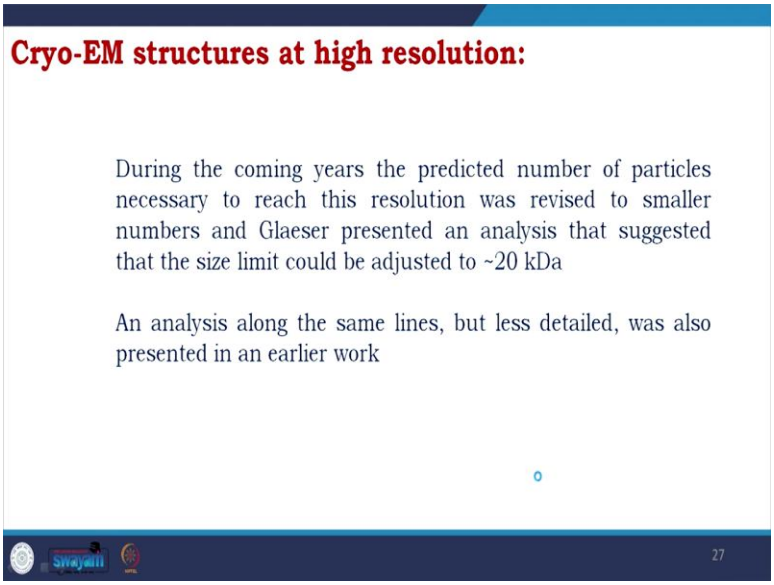


During the coming year, the predicted number of particles necessary to reach this resolution was revised to a smaller number, and Glaeser presented an analysis that suggested that the size limit could be adjusted to 20 kilodaltons. If this is possible, the cryo-electron microscope will be again getting more covering area in drug designing and all these things. Moreover, analysis along the same lines but less detailed was also present in an earlier work, which gave the possibility of lower molecular weight protein structure determination.

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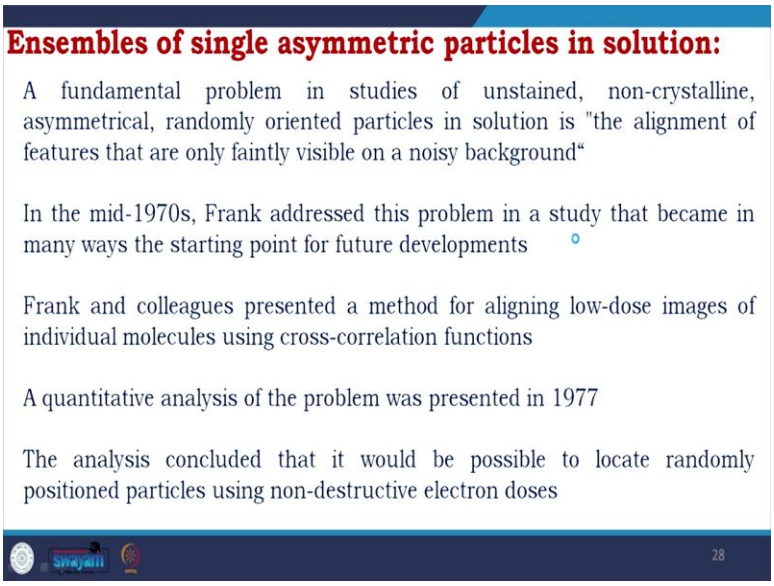


Coming to the ensembles of single asymmetric particles in solution, a fundamental problem in studies of the unstained non-crystalline asymmetrical randomly oriented particle in a solution is the alignment of the feature that is only faintly visible on a noisy background. In the mid-1970, Frank addressed this problem in a study that became the starting point of future development in many ways. Frank and his colleague Jackin Frank and his colleagues presented a method of aligning low-dose images of individual molecules using a cross-correlation function; This is an essential part of the revolution of cryo-EM. A quantitative analysis of the problem was presented in 1977. The analysis concluded that it would be possible to look at randomly positioned particles using nondestructive electron doses.

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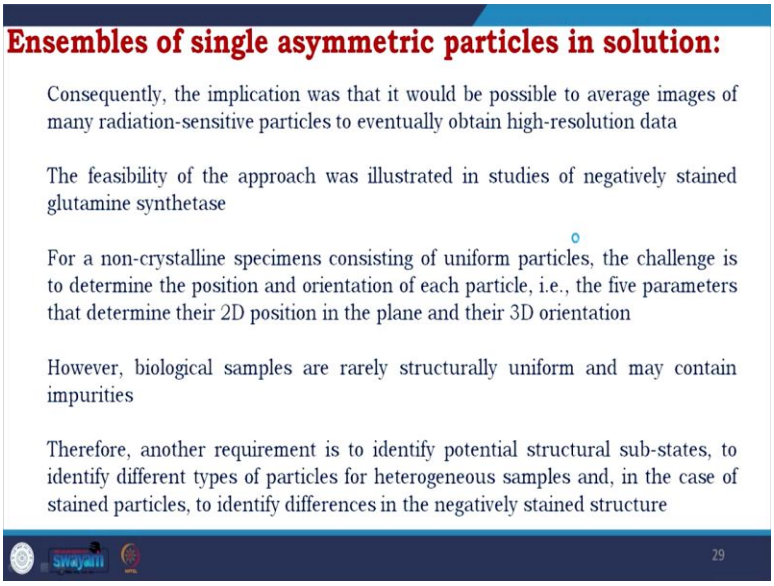

Consequently, the implication was that it would be possible to average images of many radiations sensitive particles to obtain high-resolution data eventually. The approach's feasibility was illustrated in studies of negatively strained glutamine synthetase for a non-crystalline specimen consisting of uniform particles. The challenge is to determine the position one orientation of each particle, that is, the five parameters that determine that 2D position in the plane and their 3D orientation.

So 2D position x and y and 3D orientation; however, biological samples are structurally uniform, and many contain impurities as we know. Therefore another requirement is to identify potential structural sub-states to identify different types of particles for heterogeneous samples in the case of strain particles to identify differences in the negatively strained structure. So if you look at them after negative staining, you could classify the state initially and then the data.

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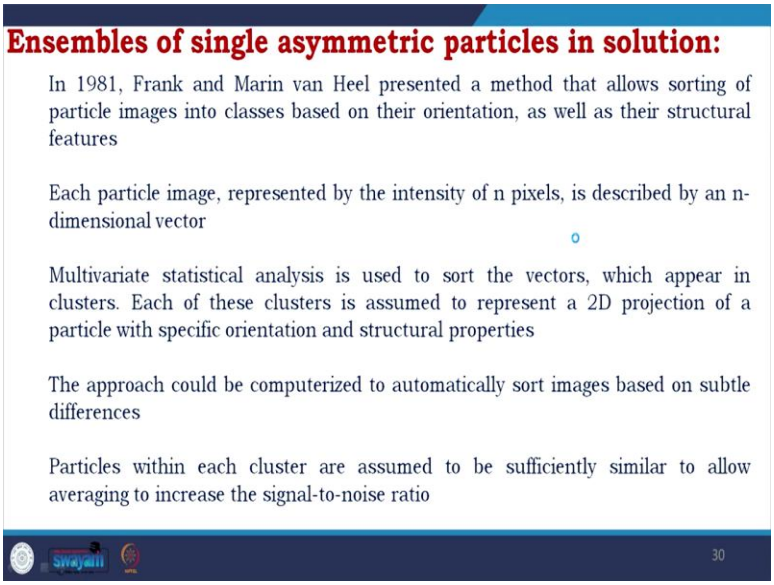


In 1981 Frank and Marin Van Heel presented a method that; allows the sorting of particle images into classes based on their orientation and structural feature. An n-dimensional vector describes each particle image represented by the intensity of n pixels. Multivariate statistical analysis is used to sort the factors which appear in clusters. Each cluster is assumed to be presented a 2D projection of a particle with specific orientation and structural properties.

The approach could be computerized to sort images based on subtle orientation differences automatically. Particles within each cluster are assumed to be sufficiently similar to allow averaging to increase the signal-to-noise ratio.

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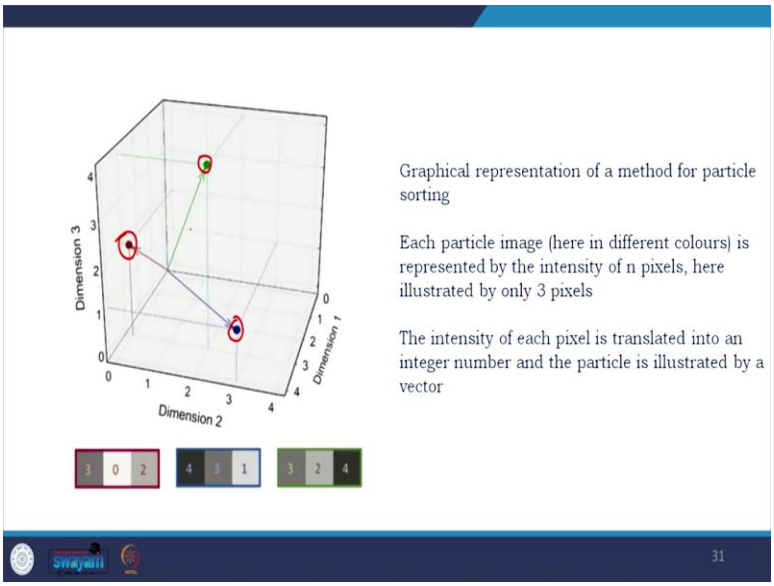


So, if you see here, it is the graphical representation of a particle sorting method. Here each particle image here it represents in three colours. One particle, two-particle, three-particle so represented by 3 pixels. The intensity of each pixel is translated into an integer number, and a vector illustrates the particle. So if you see for the rate the pixels are collected, pixels are converted into the integer and separated.

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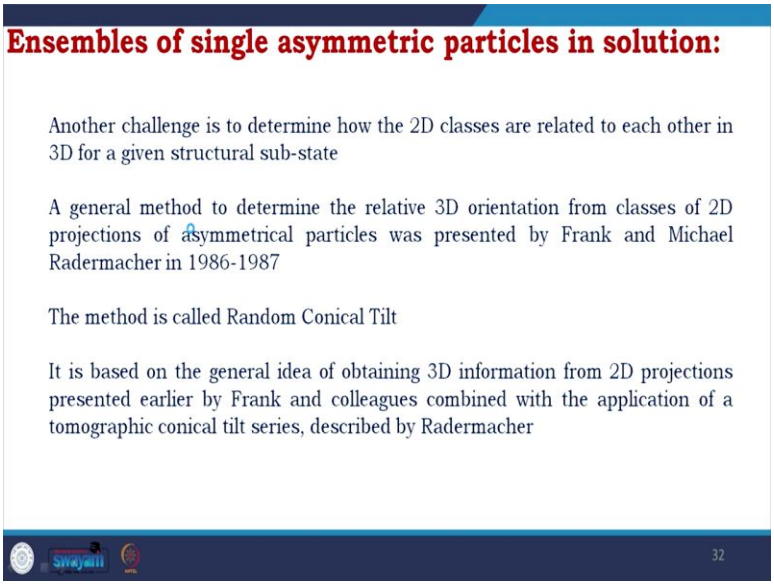

Another challenge is determining how the 2D classes are related in a 3D given structural sub-state. A general method is to determine the relative 3D orientation from classes of 2D projection of asymmetrical particles was presented by Frank and Michael Radermacher in 1986-87. The method is called Random Conical Tilt. The method is based on the general idea of obtaining 3D information from 2D projections presented earlier by Frank and colleagues combined with the application of a tomographic conical tilt series described by Radermacher.

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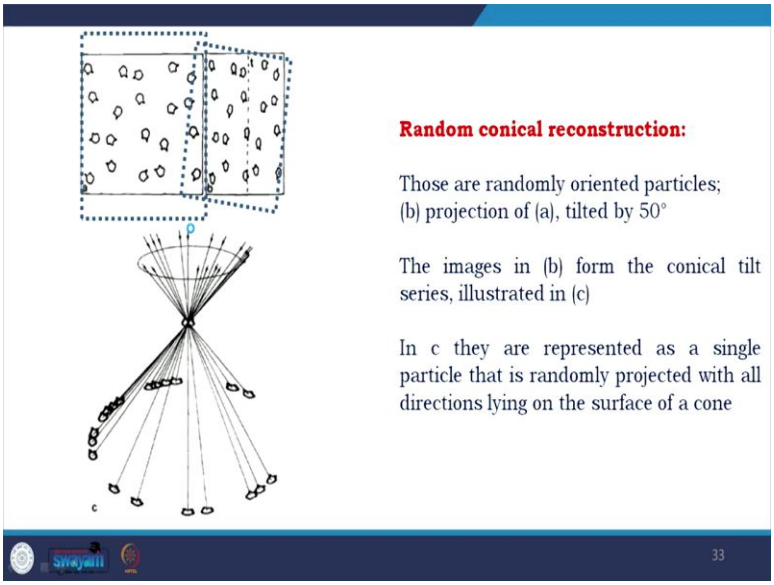


So this is the one, so ABC 3 part of the picture is called Random Conical Reconstruction. So, the randomly oriented particle in the a for projected in b by tilting 50 degrees. The images in b, which you are looking at, form the conical tilt series. In c, they are represented as a single particle that is randomly projected with all directions lying on the surface of a cone. So, that is why it is called Random Conical Reconstruction, taking from 2D doing a tilting development of a cone, giving you the projection from 2D to 3D.

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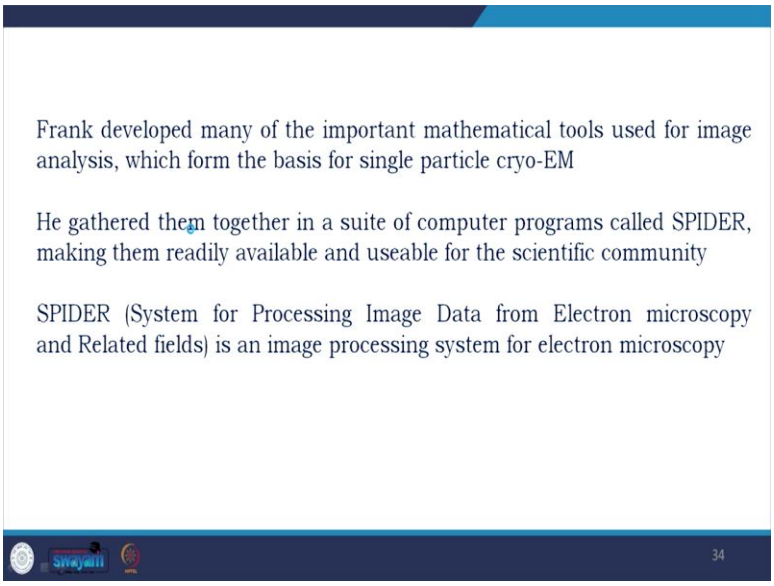


Frank developed many of the essential mathematical tools used to image analysis, which forms the basis of single-particle cryo-electron microscopy. He gets together in a suit of a computer program called SPIDER, making them available and usable to the scientific community. SPIDER is a System for Processing Image Data from Electron Microscopy and Related fields, an image processing system for electron microscopy.

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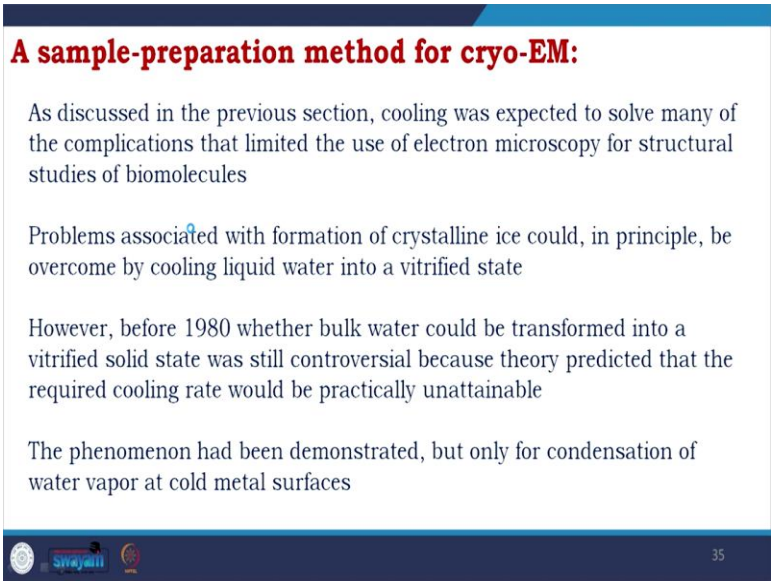


So you have already discussed the importance of sample preparation. How does the simple present happen? What are the procedures and all? As discussed in the previous section, the cooling was expected to solve many of the complications that limited the electron microscope's

use for structural studies of biomolecules, especially protein. The problem of crystalline ice formation could, in principle, be overcome by cooling liquid water into a vitrified state.

So cooling is one of the solutions for the formations of ice. With the development of vitrification, you could solve that. However, before 1980 whether bulk water could be transformed into a vitrified solid-state was still controversial because theory predicts that the required cooling rate would be practically unattainable. So, if you remember when I talk about vitrification, it is a tremendous speed. So before 1980, people thought that this was unattainable.

The phenomenon has been demonstrated but only for condensation of water vapour at cold metal surfaces.

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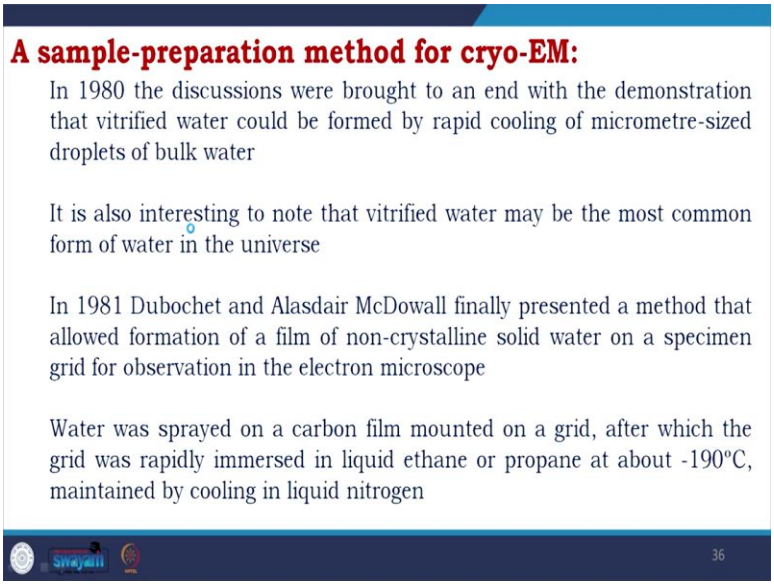


In 1980, the discussions were brought to an end with the demonstration that vitrified water could be formed by rapid cooling of micrometre size droplets of bulk water. It is also interesting to note that vitrified water may be the most common form of water in the universe. So what you want to achieve is the most common form of water. In 1981 Dubochet and Alasdair McDowell finally presented a method that allowed the formation of a film of non-crystalline solid water on a specimen grid for observation in the electron microscope.

Water was sprayed on a carbon film mounted on a grid, after which the grid was rapidly immersed in liquid ethane or propane at about -190 degrees centigrade, which is the temperature of the liquid nitrogen maintained by cooling in liquid nitrogen.

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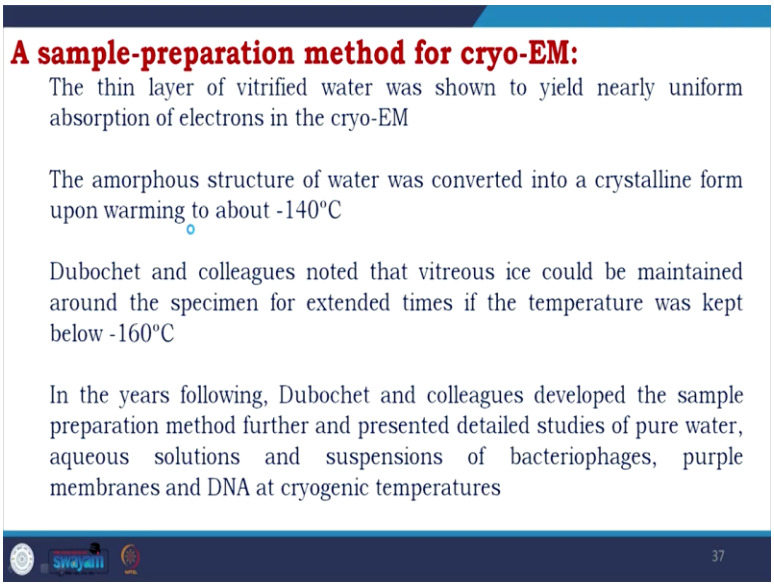


The thin layer of the vitrified water yielded nearly uniform absorption of electrons in cryo-EM, which was the dream. The amorphous structure of water was converted into a crystalline environment about minus 140 degrees centigrade Dubochet and colleagues noted that vitreous ice could be maintained around the specimen for an extended time if the temperature was kept below minus 160. So you do the freezing and maintain minus 160; you could maintain the condition.

Dubochet and colleagues developed the sample preparation method in the years following and presented detailed studies of pure water aqueous solution and suspension of bacteriophages purple membrane and DNA at cryogenic temperatures.

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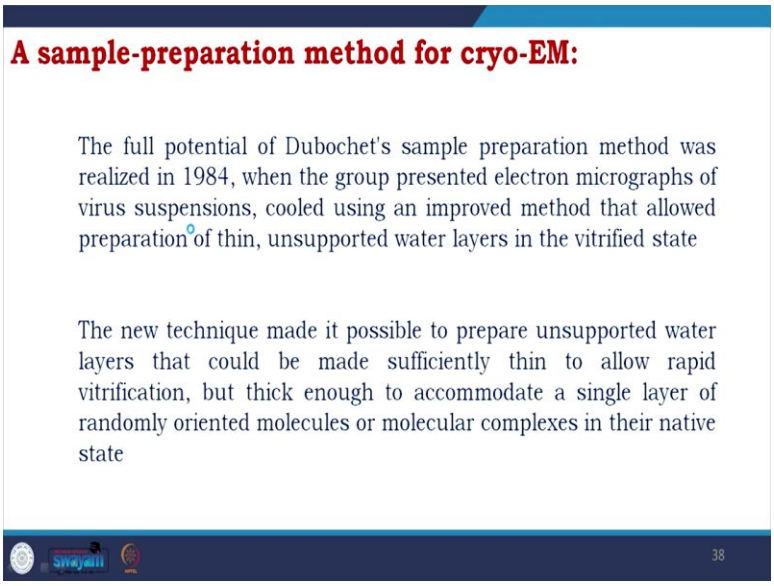


The full potential of Dubochet's sample preparation method was realized in 1984 when a group presented electron micrographs of virus suspensions cooled using an improved method that allowed the preparation of a thin unsupported water layer in the vitrified state. The new technique made it possible to prepare unsupported water layers that could be made sufficiently thin to allow rapid vitrification but thick enough to accommodate a single layer up randomly oriented molecules or molecular complexes in their native state.

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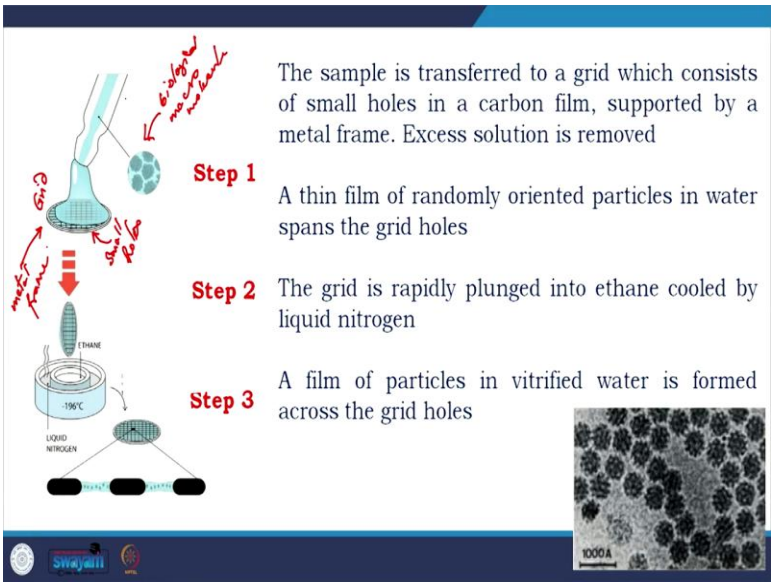


How was the sample prepared? The sample is transferred to the grid, and then the grid comes to the liquid nitrogen cooled ethane, and then the grid is coming to be prepared under the microscope. In step 1, the sample is transferred to a grid that consists of the small holes of the

carbon film. You could here; this is the grid. These are the small holes. Moreover, this is the sample where the biological macromolecules are there.

So the sample is transferred to a grid that consists of small holes in the carbon film supported by a metal frame. The excess solution is removed. I have discussed it in the preparation of sample presentation previously; when you have an excess solution, you do blotting or something, you remove it. In step 2, a thin film of a randomly oriented particle in water spans the grid holes. The grid is then rapidly plunged into the ethane cooled by liquid nitrogen.

Coming to step 3, film of the particle in vitrified water is formed across the grid holes, and you see when you see them under the microscope, what is the picture of the micrograph coming?

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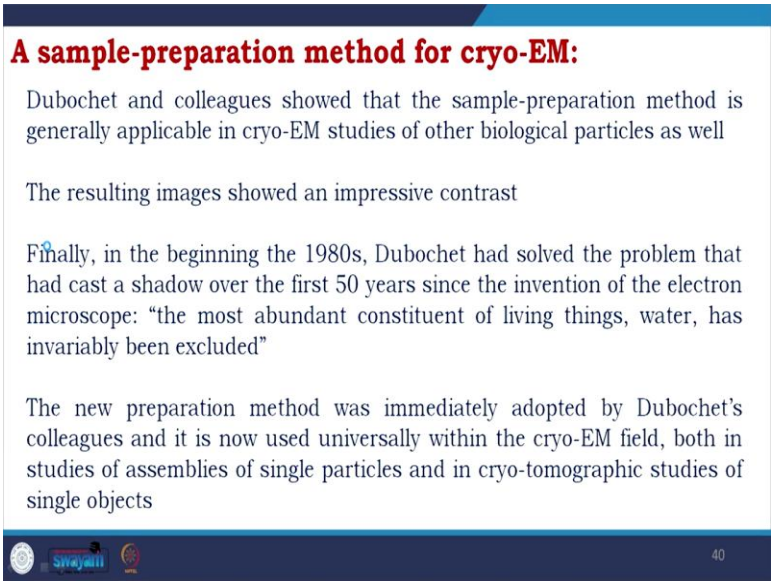


Dubochet and colleagues showed that the sample preparation method is generally applicable to cryo-EM studies of other biological particles. The resulting images should have an impressive contrast. Finally, at the beginning of the 1980s, Dubochet had solved the problem that had caused it that cast a shadow over the first 50 years since the invention of the electron microscope. The most abundant constituent of living things, water, has invariably been excluded.

So this is the final step. Dubochet and colleagues immediately adopted the new preparation method, and it is now used universally within the cryo-EM field both in studies of assemblies of single-particle and cryo-tomographic studies of a single object.

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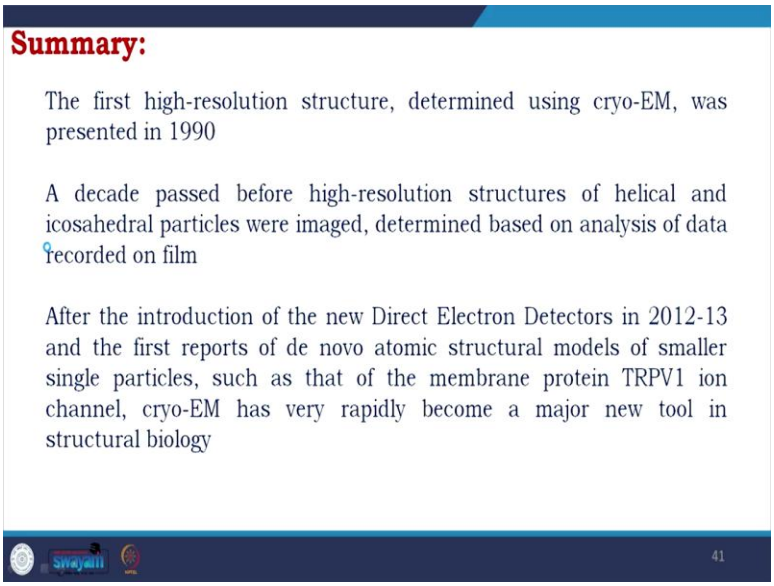


So that is all about the process details coming to the summary. The first high-resolution structure determined using Cryo-EM was present in 1990 the structure of bacteriorhodopsin by Henderson. A decade passed before the high-resolution structure of helical and icosahedral particles was imaged and determined based on data analysis recorded on film. After introducing the new direct electron detector in 2012-13, one of the landmarks and the first reports of de novo atomic structural model of smaller single particle such as that for membrane protein TRPV1 ion channel. Cryo-EM has rapidly become an essential new structural biology tool for determining atomic structure.

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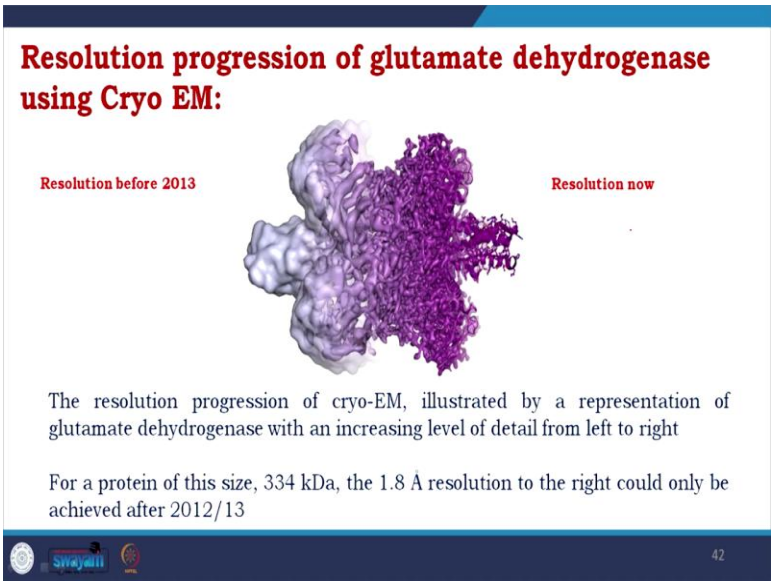


You could see here the structure of glutamate dehydrogenase, one of the stable proteins that take much time to make the micrographs and analyze. So these bloody structures were resolved before 2013, which is improved now, and you could easily see the atomic-level structure. So, the resolution progression of cryo-EM is illustrated by the representation of glutamate dehydrogenase with an increasing level of detail in the left to right.

For a protein of this size 334 kilodalton, the 1.8 resolution to the right could only be achieved after 2013.

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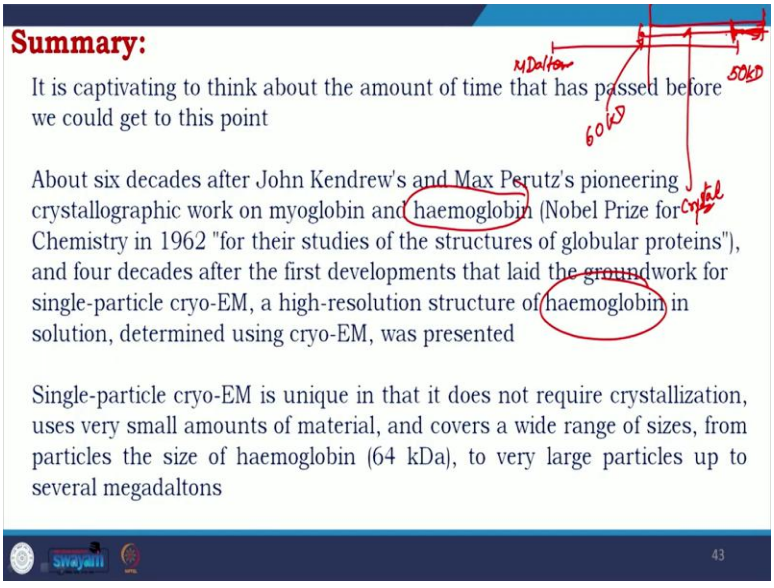

It is captivating to think about the amount of time before we could get to this point—about six decades after John Kendrew's and Max Perutzu's crystallographic work and myoglobin and haemoglobin. As discussed in detail, the Nobel Prize for Chemistry in 1962 for the studies of the first structure of the globular protein and four decades after the first development of the lid that laid the groundwork for single-particle cryo-EM, a high-resolution structure of haemoglobin.

So haemoglobin in the first structure comes in a solution determined using cryo-EM. Single-particle cryo-EM is unique because it does not require crystallization; as I told you for a long time, this criterion uses a very small amount of material that covers a wide range of sizes from a particle the size of haemoglobin 64 kilodaltons to very large particle up to Megadalton. So at that point, you could compare NMR crystallography and cryo-EM.

Cryo-EM can solve from more than 50 kilodaltons to several megadalton cryo-EM. X-ray kind covering from low to this if you have a crystal NMR is limited to 60 kilodaltons till now 60 kilodaltons, so where X-ray starts NMR ends. So this is one of the excellent comparisons from small peptide to 60 kilodaltons NMR is suitable. For any peptide to a good protein size to a few 100 kilodaltons, the x-ray is good.

From 50 kilodalton to several kilodaltons, cryo-EM is good—however, cryo-EM works with the native condition identifying the confirmation and all in the atomic level. X-ray and crystal NMR need a very high protein concentration.

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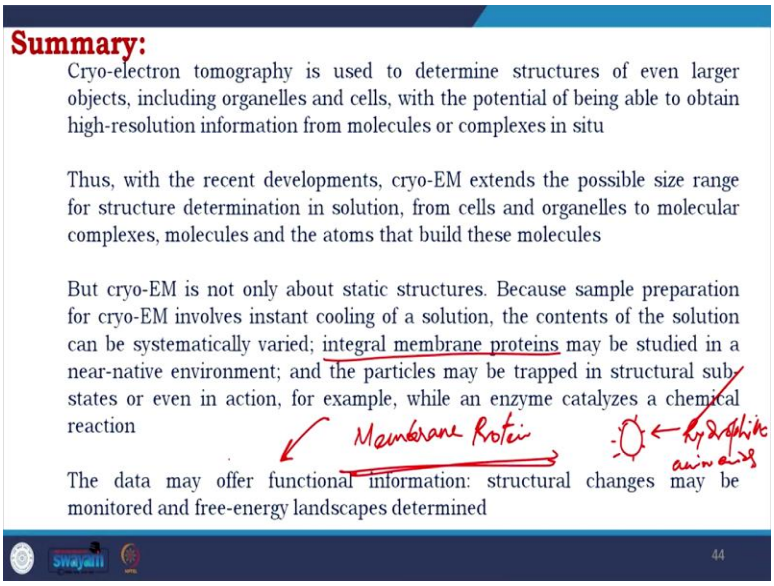


Cryo-electron tomography is used to determine the structure of even larger objects, including organelle cells, with the potential to obtain high-resolution information from molecules or complexes in situ. Thus with the recent development, cryo-EM extends the possible size range for structure determination in solution from cells and organelles to molecular complexes, molecules and atoms that build these molecules, but cryo-EM is not only about static structures because sample preparation for cryo-EM involves instant cooling of a solution, but the contents of the solution can also be systematically varied; integral membrane protein may be studied in a near-native environment, and the particles may be trapped in structural substrate or events in action, for example, while enzyme catalyzes chemical reaction a lot of biological conditions could be trapped that is the potential of the upcoming instrument.

Also, one thing I have to repeat about membrane protein. As you already understood, soluble proteins have hydrophilic amino acids. Hydrophilic amino acids form hydrogen bonds with water and solubilize for the membrane protein. The hydrophobic residues at the surface could not be solubilized. So to solubilize, they need perfect conditions, which is why it was challenging to work in both crystallography and NMR.

You could directly get the membrane, freeze it and get the structure. Membrane protein and related development are possible with the advent of electron microscopy, a high-resolution cryo-

electron microscopy. Data may offer functional information, structural changes may be monitored, and the free energy landscape could also be determined.

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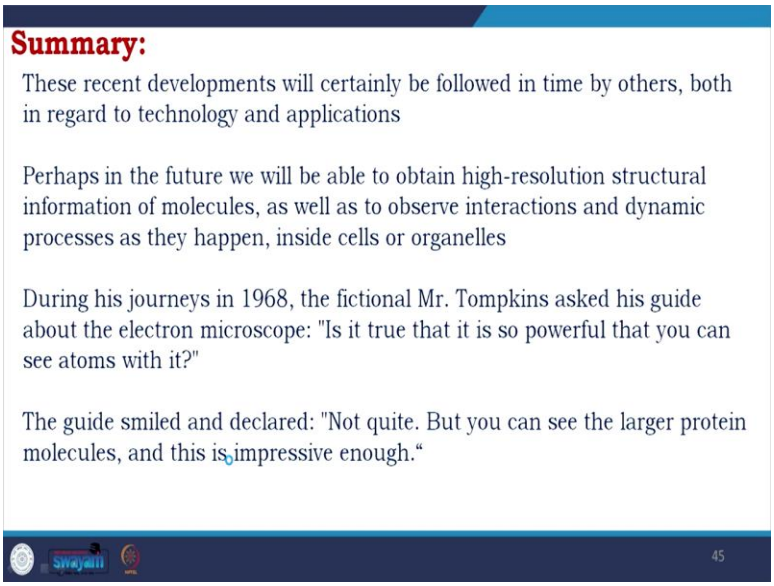


Others will undoubtedly follow this recent development regarding technology and applications. Perhaps in the future, we will be able to obtain high-resolution structural information of molecules and observe the interaction and dynamic processes as they happen inside the cells or organelles; probably, you could get the entire cell's functional mechanism, which would be amazing.

During his Journeys in 1968, we are going back. Now the fictional Mr Tompkins asked his guide about the electron microscope. Is it true that it is so powerful that you can see atoms with it? The guide smiled and declared not quite, but you can see the larger protein molecules, impressive enough. A question and related answer, a doubt, very interestingly the whole scenario was coming in the life of Professor Henderson, and we will finish our story talking about that.

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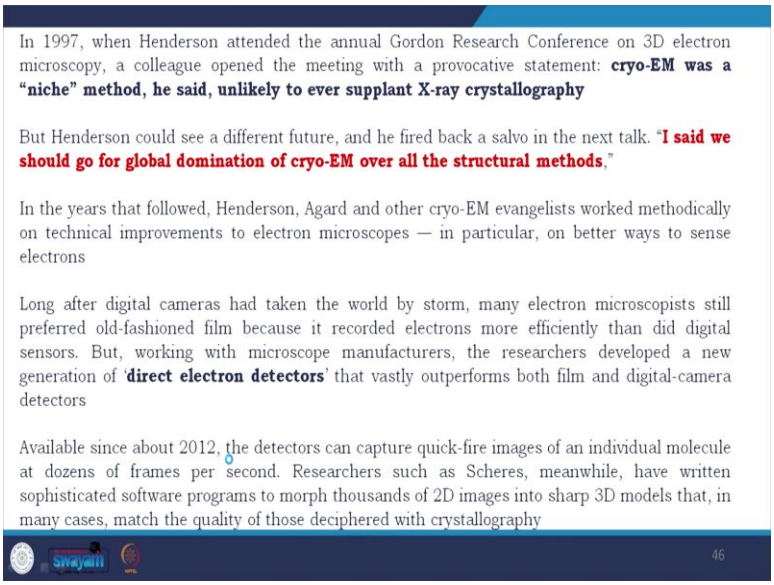


In 1997 when Henderson attended the Annual Gordon Research conference on 3D electron microscopy, a colleague opened the meeting with the provocative statement. Cryo-EM was a "niche" method; he said it was unlikely ever to supplant the X-ray crystallography. However, Henderson could see a different future, and he fired a salvo in the next talk. "I said we should go for global domination of cryo-EM over all the structural methods".

In the year that followed, Henderson, Agard and other cryo-EM evangelists worked methodically on technical improvements to electron microscope in particular and better ways to sense electrons. Long after the digital camera took the world by storm, the researcher developed a new generation of direct electron detectors that first outperformed film and digital camera detectors.

So the innovation of direct electron detectors, as I have told during this course for some time, is one of the keys. Since 2012, the detector can capture quick-fire images of an individual molecule at dozens of frames per second. These have given cryo-electron a very significant move.

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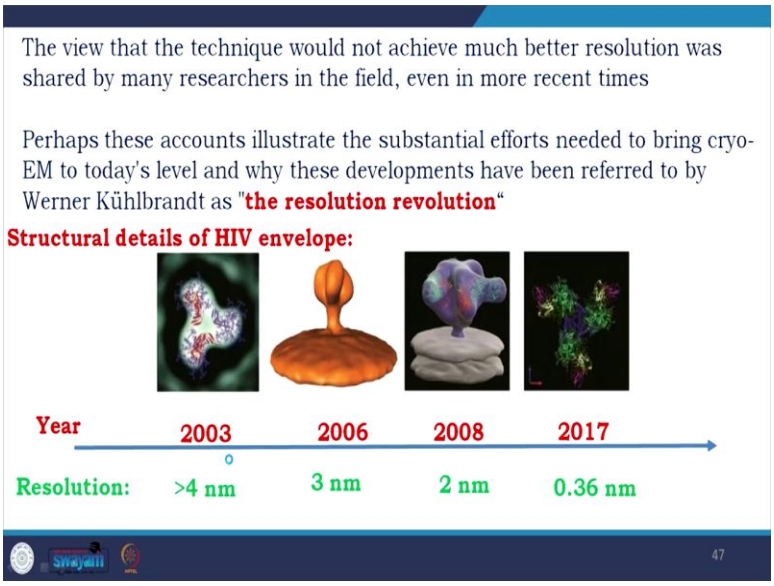


Many researchers about cryo-EM shared that the technique would not become active much better resolution. Perhaps this account illustrates the substantial effort needed to bring cryo-EM to today's level and why Werner Kuhlbrandt has referred to this development as the resolution revolution. I am showing you than many, many like examples nowadays, but I will show you the structural details of the HIV envelope; you will see that the resolution becomes higher and higher in 2003 - 2006 2008 - 2017.

The first one was more significant than 4 nanometers in 2003 it came to 3 nanometers in 2006. It comes 2, nanometer at 2008 and 0.36, NM 3.6 angstrom at 2017.

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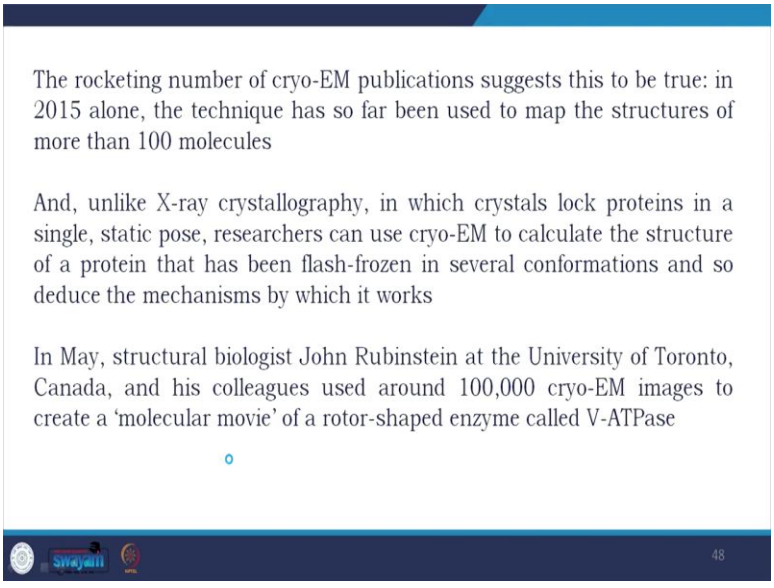

Rocketing number of cryo-EM publications suggest this to be true. In 2015 the technology was used to map the structure of more than 100 molecules, but in 2018, it even surpassed the number of structural entries in a single year to the number NMR has achieved. So that is the significant one. Furthermore, unlike x-ray crystallography in which crystals of proteins in a single static pose, researchers can use cryo-EM to calculate the structure of a protein that has been flash-frozen in several conformations and deduce the mechanism of how it works.

So crystallography gives you a high-resolution structure, but cryo-EM is talking about the biology and now in exact resolution. In May, structural biologist John Rubinstein at the University of Toronto and his colleagues used around 100 000 cryo-EM images to create a molecular movie of a rotor shaped enzyme called V-ATPase.

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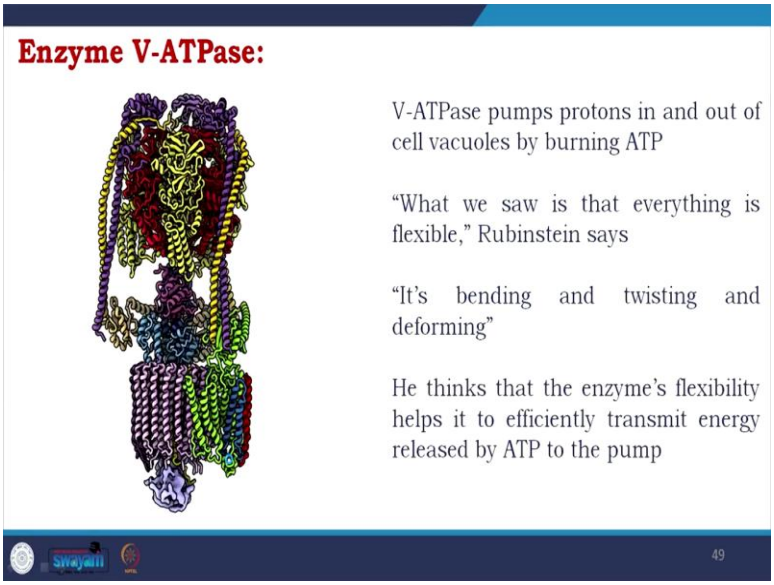


So this is enzyme V-ATPase, and this is the molecular movie they developed by taking 1 lakh micrographs. So it is not only now with 3D development. It is 3D movement development. That is also added here in the cryo-electron microscopy technique, giving us more understanding. So, V-ATPase pumps protons in and out of the cell vacuole by burning ATP. What we saw is that everything is flexible. Looking at the protein, you will understand what Rubinstein says: Everything is flexible, which is a new mode of vision we are getting by advancing the electron microscope technique.

It is bending, twisting and deforming. The enzymes flexibility helps it transmit energy released by ATP to the pump efficiently. So, now from a high-resolution structure, you could also go for our determination of understanding of function directly.

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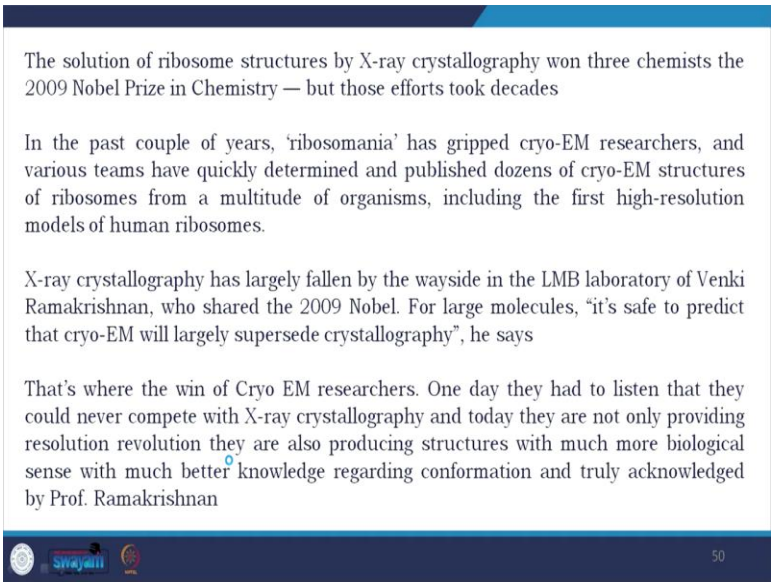


The solution of ribosome structure by x-ray crystallography on three chemists, the 2009 Nobel Prize in chemistry Venki Ramakrishnan is one of them. Thomas Steel got a Nobel Prize for the ribosome structure, but in addition to that, the ribosome has gripped cryo-EM structures; why? Because ribosomes are a stable complex and are good enough in size to go for cryo-electron microscopy, it is crucial because it is a protein synthesis machinery.

So various people teamed up quickly and determined and published dozens of cryo-electron microscopic structures of ribosomes from many organisms, including the first high-resolution model of the human ribosome. As I told you, x-ray crystallography has largely fallen by the wayside in the LMB laboratory of Venki Ramakrishnan, who share the 2009 Nobel. He says that large molecules shape predict that the cryo-EM will largely supersede crystallography.

That is where the win of cryo-electron microscope researchers. One day they had to listen that they could never compete for supersede x-ray crystallography. Today, they provide the resolution at the level of x-ray crystallography and produce structures with a much more

biological sense, with a much better knowledge regarding confirmation regarding activity regarding its connection to biology.

Moreover, now it is genuinely acknowledged by Venky crystallographers that this technique has gone so far and now established itself as one of the most well-known structure solution techniques.

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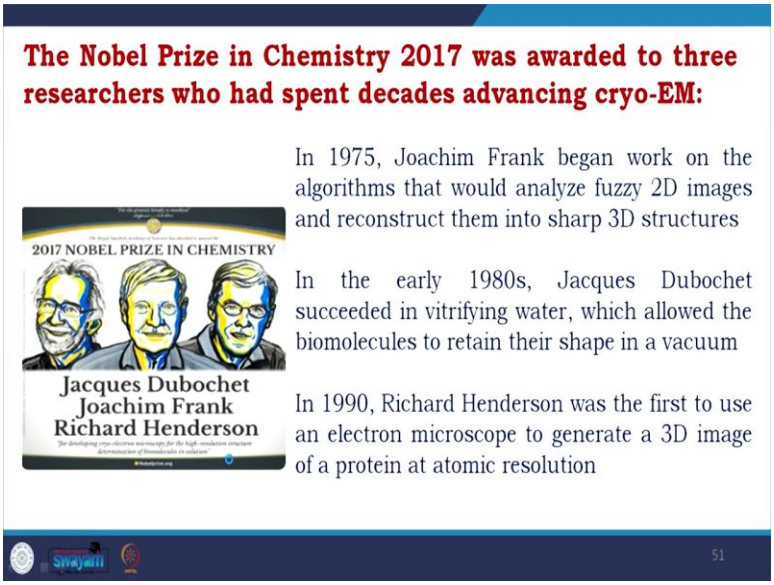


With that, I would remember their Nobel Prize Chemistry work of them. So as I am already talked about, the Nobel Prize in chemistry 2017 was awarded to three researchers who had to spend decades advancing cryo-electron microscopy. You all know that history now, what they did with talked in details, but they summarised in 1975 Joachim Frank began work on the algorithm that will analyze detailed 2D images and reconstruct them into the sharp 3D structure.

So, from a 2D micrograph picture to a model. In the early 1980s, Jacques Dubochet succeeded in vitrifying water, which allowed the biomolecules to retain shape in a vacuum, so Jacques Dubochet did the vitrification of water. In 1990 Richard Henderson was the first to use an electron microscope to generate a 3D image of a protein at atomic resolution. So that is about cryo-electron microscope and their advancement. Moreover, I have compared each level with the other two techniques.

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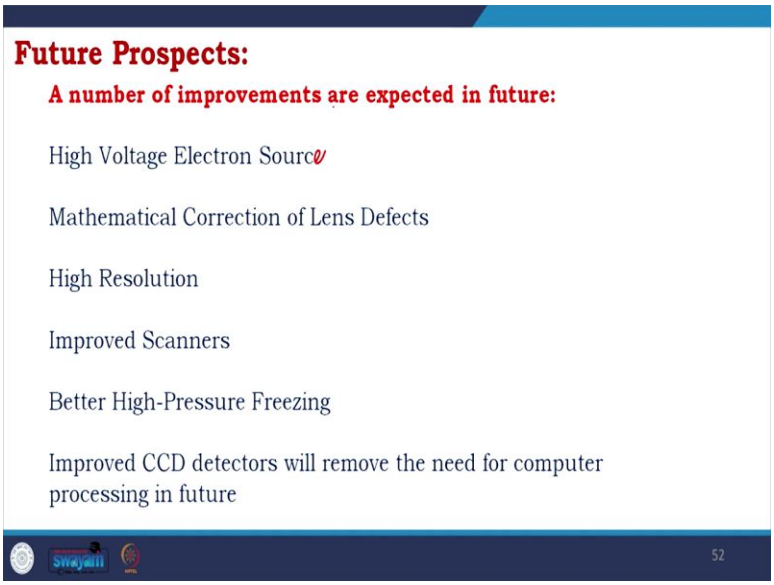


I will finish up with the prospects in the number of improvements are expected in future High Voltage Electron Source, Mathematical Correction of Lens Defect, the aberrations. Higher resolution, Improved Scanners, Better High-Pressure Freezing which is one of the places much work is needed and Improved CCD or direct electron detectors will remove the need for computer processing in future. So that is about electron microscopy, as I have told you earlier.

This is not a course where I am teaching you cryo-electron microscopy. The idea of this course is to make you knowledgeable about Structural Biology. You should know about the basics of this technique, and I tried to communicate, try to make you excited, and I am ending up saying look at this technique; this is a technique for you to look at because this a technique which will contribute significantly to the world of Structural Biology, thank you very much.