Structural Biology
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Lecture – 37
Functioning Details of Cryo-Electron Microscopy (Cryo-EM)

Hello everyone, welcome again to the course on structural biology. We are continuing with structural biology techniques. In the last class of the module of cryo-electron microscopy, we start with a generalized idea about microscopy. We have introduced cryo-electron microscopy but talk more about light microscopy, electron microscopy, some history and detailing of electron microscopy. Today we will come to the topic directly for which we are making this module the cryo-electron microscopy.

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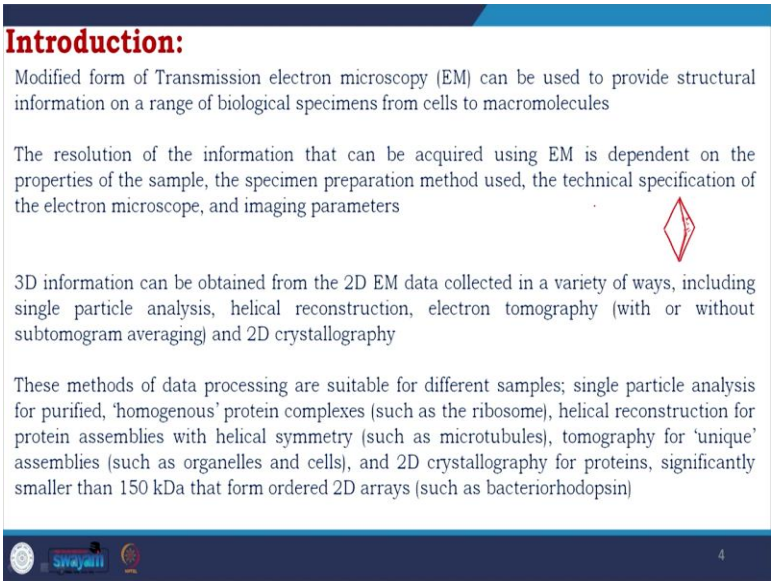

Cryo-electron microscopy could be divided into cryo-transmission electron microscopy and scanning electron microscopy. The original cryo-electron microscopy is coming through cryo-electron transmission microscopy; the transmission electron microscopy could be divided into single-particle and cryo-electron tomography. We will talk about them and then discuss the instrumentation.

So, as I told it is a modified form of transmission electron microscopy, and it can be used to provide structural information on a range of biological specimens from cells to macromolecules;

This is very important when we are talking about x-ray crystallography; x-ray crystallography is specific for DNA, RNA, protein and viruses. NMR has many dimensions that could identify small molecules; they could identify secondary structures.

Many different types of experiments could be employed for dynamic studies and used for getting the metabolic profile, and there are many uses. In cryo-electron microscopy, they could also get the details of cells and relatively larger assemblies. Even in macromolecules, a cryo-electron microscope is not good for small proteins, but it is good for bigger assemblies; we will talk about them.

The resolution of the information that can be acquired using electron microscopy is dependent on the properties of the sample, the specimen preparation method used, the technical specification of the electron microscope and imaging parameters. So, the properties of the sample are one determinant specimen preparation method. We will talk about the technical specification of the electron microscope and imaging parameters.

3D information can be obtained from 2D electron microscopy data collected in various ways, including single-particle analysis, in which I talked about helical reconstruction, electron tomography with or without sub tomogram averaging and 2D crystallography. These data processing methods are suitable for different samples, single-particle analysis for purified homogeneous protein complexes, mostly the work done with the ribosome nuclear pore system.

Helical reconstruction for protein assemblies with helical symmetry such as microtubules tomography for unique assemblies such as organelles and cells and 2D crystallography of protein significantly smaller than 150 kilodaltons that form ordered 2D arrays such as bacteriology. So, upon crystallization, when they are not forming a 3D crystal. This axis only forms flat crystals, a 3D crystal, and this is a 2D crystal. So, the 2D crystal could also be solved by using cryo-electron microscopy.

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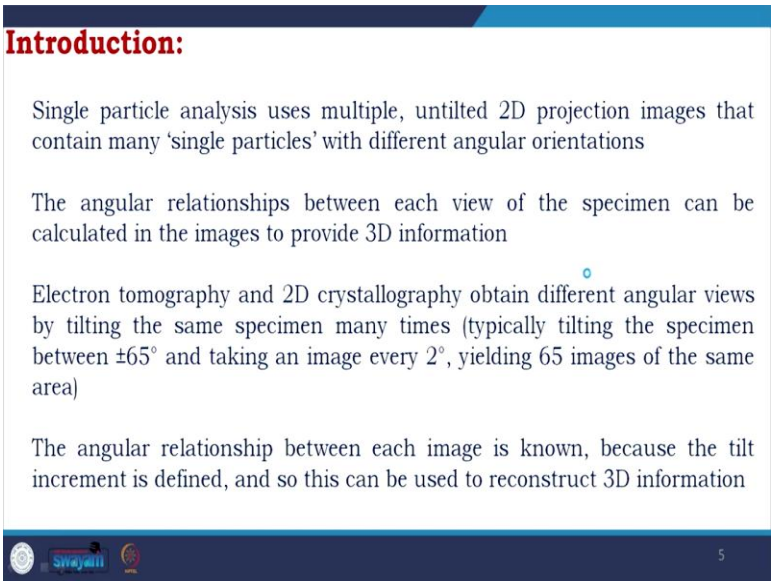

The single-particle analysis uses multiple and tilted 2D projection images that contain many single particles with different angular orientations. So, you have collected different images with different angular orientations; you have to work on them. The angular relationship between each view of the specimen can be calculated in the images to provide 3D information. Electron tomography and 2D crystallography obtain different angular views by tilting the same specimen many times, typically tilting the specimen between plus-minus 65 degrees and taking an image every 2 degrees yielding 65 images of the same area.

The angular relationship between each image is known because the tilt increment is defined, and so, this can be used to reconstruct 3D information; we will talk about these in detail in the data analysis section.

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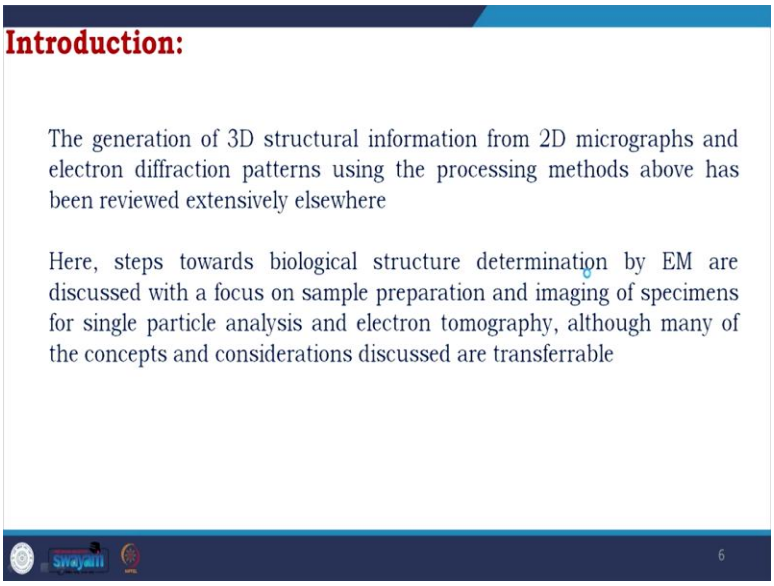

The generation of 3D structural information from 2D micrographs and electron diffraction patterns has been reviewed extensively using processing methods. Later we will primarily focus on the general simplified method and its derivative. Here, steps towards biological structure determination by electron microscopy are discussed, focusing on sample preparation and imaging of specimen for single particle analysis and electron tomography. However, many of the concepts and considerations we have discussed apply to generalized other systems.

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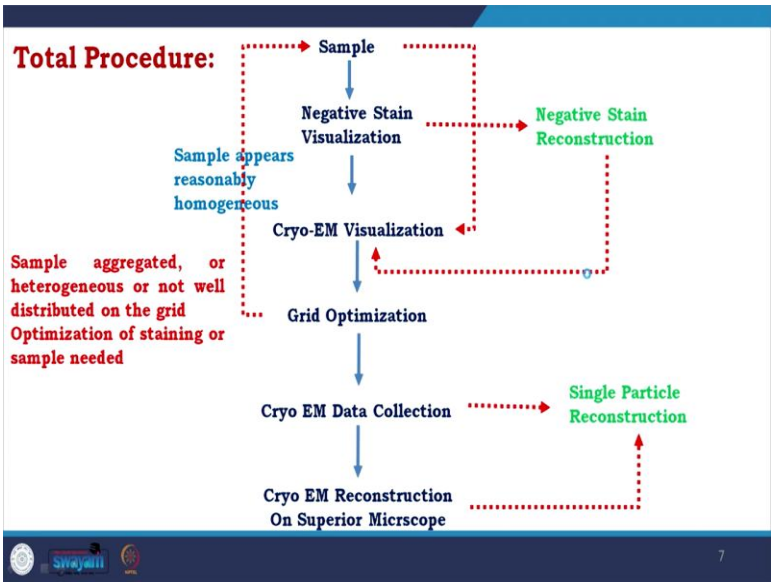

So, let us first look at the whole procedure by which cryo-electron microscopy works. First, you have a sample then you have to stain the sample with a negative stain for the visualization. So, you take the sample, do the negative strain, and visualize it. Then you go for cryo-EM

visualization you make the optimization of the grid. Grid is the platform we will discuss where you will put your sample.

Then you will do the cryo-electron microscopy data collection, and ultimately you will do cryo-EM reconstruction on a superior microscope high-resolution microscope. So, these are the basic steps: sample, negative strain visualization, cryo-electron visualization, grid optimization, and cryo-electron data collection and reconstruction. However, if the sample appears reasonably homogeneous, homogeneity is a critical component of successfully doing this experiment.

Now you could go directly without negative strain; you go to cryo-EM visualization directly. You could also make negative strain reconstruction and then go to cryo-EM visualization if your sample aggregate is heterogeneous or is not well distributed on the grid optimization for staining or the sample. So, what are the conditions for your sample to get aggregated?

If it is not homogeneous, it shows heterogeneity. If it is not well distributed on the grid, your staining is not optimized, or your sample needs to be optimized, you have to check the sample in grid optimization state before the data collection then back for preparing the samples. Also, from cryo-electron data collection, you could go for single-particle reconstruction.

So, we will talk about sample preparation, negative staining, visualization grid optimization and data collection in this class.

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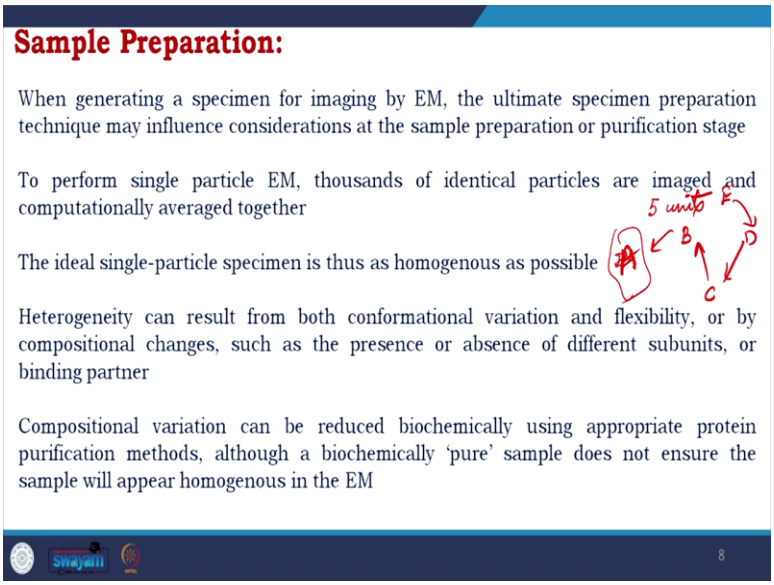

Coming to sample preparation, when generating a specimen for imaging by electron microscopy, the ultimate specimen preparation technique may influence consideration at the sample preparation or purification stage. To perform single-particle electron microscopy, thousands of identical particles are imaged and computationally averaged together. The ideal single-particle specimen is thus as homogeneous as possible.

Heterogeneity can result from both conformational variation and flexibility or by compositional changes such as the presence or absence of different subunits or binding partners. So, if you are going to construct scenarios where you have a complex, you know these guys are making these complexes, and you want to see that in the absence of like you have five units or five binding partners, and one is big enough to start with single-molecule electron microscopy.

So, you put molecule A and then you add B, then you add C, then you add D, then you add E. In that way, there would be changing composition of the subunits binding partner changing the homogeneity of the solution; This is not good for single particular microscopy. If you have to do single particulate microscopy, you have to make a stable unit and then use this homogeneous unit. Compositional variation can be reduced biochemically using the appropriate protein purification method.

We have talked about different purification methods. We have talked about affinity chromatography and size exclusion chromatography principles. We have already talked about them in detail, and we have also talked about ion-exchange chromatography. So, these are different methods we have discussed in detail in x-ray crystallography, but you have to apply the appropriate protein purification method.

Although a biochemically pure sample does not ensure the sample will appear homogeneous. So, how do you know a biochemically pure sample: you will run in the SDS gel and get a pure band. So, you know it is pure protein, but when you do silver staining, you will see that this protein is not so pure. So, the definition of a biochemically pure protein differs. The protein might have two conformations.

So, when you are putting it into SDS, you denature the protein by applying SDS and all it shows one band, but in actuality, it has different conformations. So, many things are possible.

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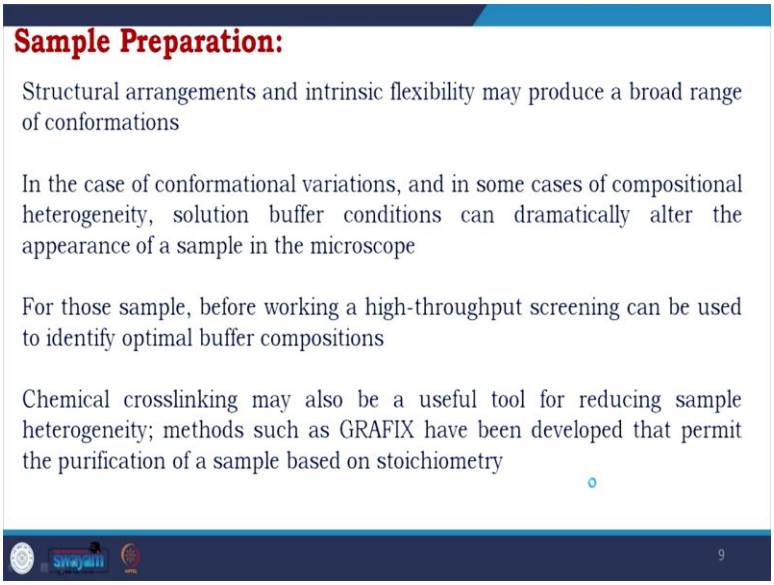

Structural arrangements and intrinsic flexibility may produce a broad range of conformation. So, if your protein is structurally flexible, this is the intrinsic property of the protein; it could produce different conformations. In the case of conformational variation and some cases of compositional heterogeneity, solution buffer conditions can dramatically alter the appearance of a sample in the microscope.

So, in those conditions to bring the homogeneity, which is a critical condition to make the cryo-electron microscopy experiment successful, one has to screen with different buffers and see dramatic changes in the homogeneity profile of the protein. For those samples before working, a high throughput screening can be used to identify optimal buffer compositions. Chemical cross-linking may also be a helpful tool for reducing sample heterogeneity.

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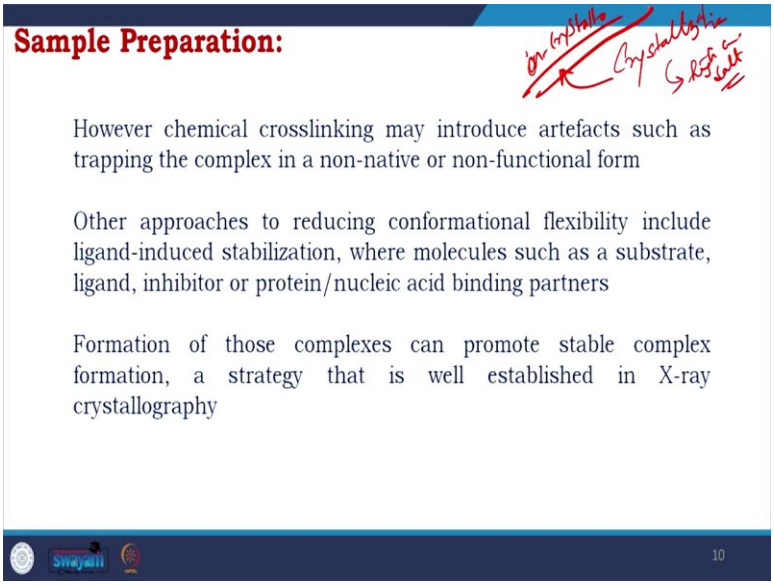

However, the chemical cross-linking may introduce artefacts such as trapping the complex in a non-native or non-functional form. So, as I was talking from the initial stage, the structure must make correlations between the sequence and function. However, to do that, the structure has to show the information about the native state, and that is one of the problems we are always discussing in the case of crystallography.

We do the crystallization in a very high concentration of salt and all, and we call this condition a non-native condition; we call it in-crystallo condition. Your chemical cross-linking might introduce artefacts, and we do not want to do that. However, sometimes the complex is so unstable that we have to do it.

It is always a loss and gains problem like you have to keep trying. Other approaches for reducing conformational flexibility include ligand-induced stabilization, which we talked about when

ligand comes ligand to bind with the protein and stabilize it where molecules such as a substrate ligand inhibitor or protein-nucleic acid binding partners. The formation of those complexes can promote stable complex formation.

As I talked about a strategy that is well established in extra crystallography already, we talked about this stabilization factor when we talked about the x-ray.

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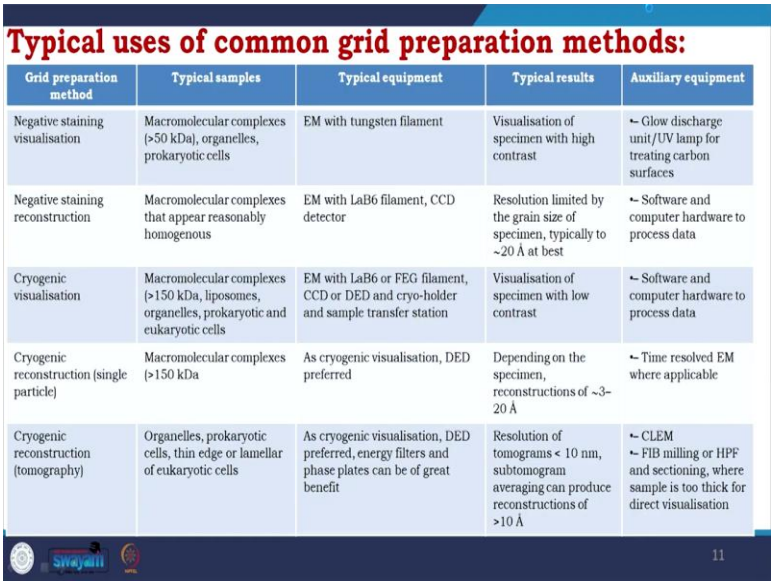

Typical uses of common grid preparation method there are different one. Negative straining visualization is a grid preparation method. So, you have different methods negative staining visualization, negative straining reconstruction, cryogenic visualization, cryogenic reconstruction when you go to a single particle, cryogenic reconstruction in the case of tomography. So, for negative straining visualization, the typical samples are macromolecular complexes greater than 50 kilodalton organelles prokaryotic cells.

For negative staining reconstruction, macromolecular complexes that appear reasonably homogeneous for cryogenic visualization macromolecular complexes more significant than 150 kilodalton liposomes, organelles, prokaryotic and eukaryotic cells. For cryogenic reconstruction for single-particle macromolecular complexes greater than 150 kilodaltons, for cryogenic reconstruction, tomography organelles prokaryotic cells thin age or lamellar of eukaryotic cells.

Typical equipment for negative straining visualization electron microscopy with tungsten filament, negative staining reconstruction electron microscopy with LaB6 filament CCD detector cryogenic visualization EM with LaB6 or FEG filament we would talk about them. CCD or DED and cryo holder and sample transfer stations. For cryogenic reconstruction as cryogenic visualization, DED is preferred. Cryogenic reconstruction as cryogenic visualization DED preferred.

Energy filters and phase plates can be of great benefit-typical results for negative straining visualization specimen visualization with high contrast. For negative straining, reconstruction resolution is limited by the grain size of b the specimen, typically to 20 angstroms at rest. Cryogenic visualization, the typical result is the visualization of the specimen with low contrast. Cryogenic reconstruction for single-particle depending on the specimen reconstruction of 3 to 20 angstrom we are going towards high resolution.

Cryogenic reconstruction for tomography resolution of Tomographs less than 10 nanometers; Sub tomogram averaging can produce a reconstruction of around 10 angstroms. Auxiliary equipment for negative straining visualization, glow discharge unit, UV lamp for treating carbon surfaces, negative staining reconstruction software and computer hardware to process data, cryogenic visualization software, and com hardware to process data.

Cryogenic reconstruction time-resolved electron microscopy will discuss this in the next class in spatial instrumentation. Cryogenic reconstruction CLEM is also a special type of laser electron microscopy; we will discuss FIB milling or HPF and sectioning where the sample is too thick for direct visualization.

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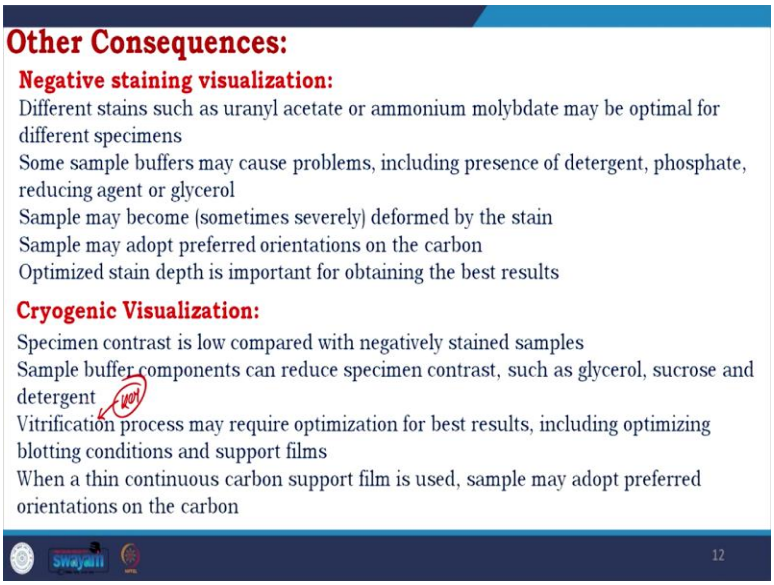

Other consequences which are of special mention for negative straining visualization different strains such as urinal acetate or ammonium molybdate may be optimal for the different specimen. Some sample buffers may cause problems, including detergent phosphate reducing agent or glycerol. The sample may become deformed by the stain, which sometimes has a severe effect. The sample may adopt a preferred orientation on the carbon; optimized chain depth is essential for obtaining the best results.

For cryogenic visualization, specimen contrast is low compared with negatively stained samples. Sample buffer components can reduce specimen contrast, such as glycerol, sucrose and detergents. Vitrification: vitrification process may require optimization for best results, including optimizing blocking conditions and support films.

When a thin continuous carbon support film is used sample may adopt preferred orientation on the carbon. So, we sometimes need the thin continuous carbon support film when it does not get the optimized conformation.

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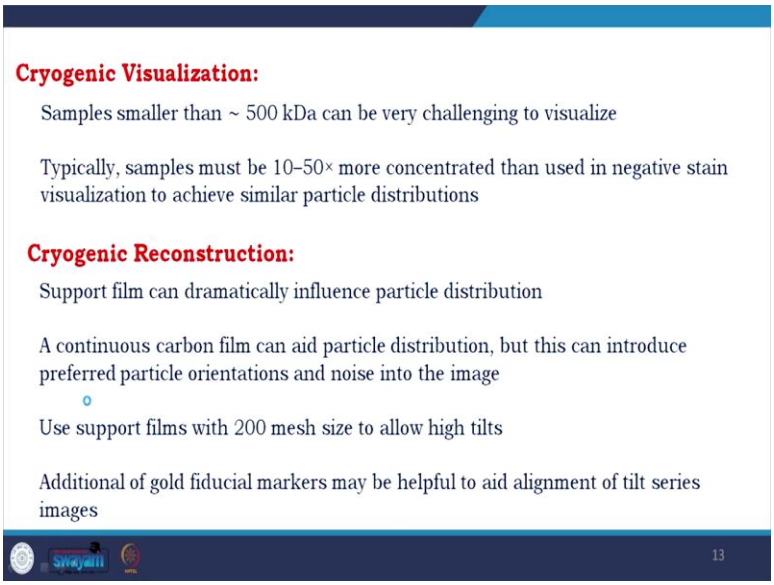

Samples smaller than 500 kilodaltons can be very challenging to visualize with cryogenic visualization. Typically sample must be 10 to 50x more concentrated than used in negative strain visualization to achieve similar particle distribution. Cryogenic reconstruction for single-molecule support film can dramatically influence particle distribution. So, support film is essential. A continuous carbon film can aid particle distribution, introducing preferred particle orientation and noise into the image.

For tomography, use support films with 200 mesh size to allow high tilt. Additional gold fiducial markers may be helpful to add ailment of tilt series images when you are going to collect the tilt images, calculate the angular positions and reconstruct the 3D image.

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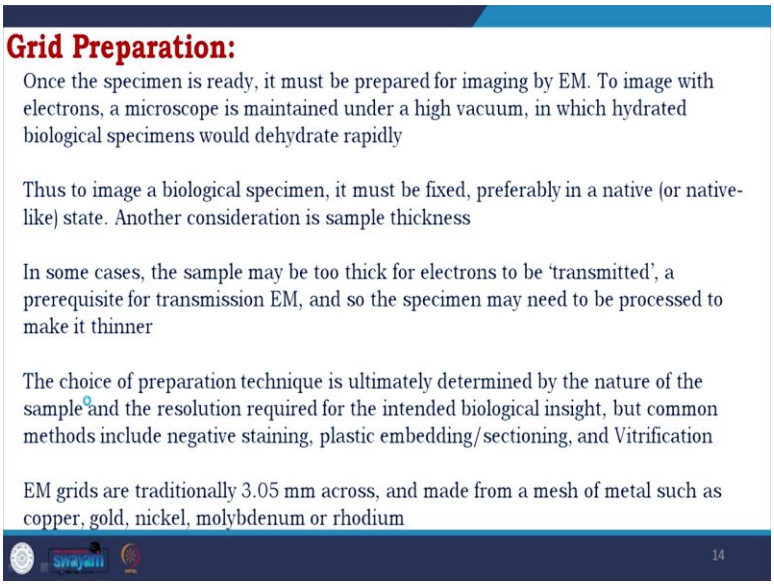

The grid must be prepared from imaging by electron microscopy once the specimen is ready. For imaging with an electron microscope, it should be maintained under a very high vacuum in which hydrated biological specimens would dehydrate rapidly. So, this is a huge problem. Thus to image a biological specimen, it must be fixed, preferably in a native or native-like state. Another consideration is sample thickness.

In some cases, the sample may be too thick for electrons to be transmitted, a prerequisite for transmission electron microscopy, and so, the specimen may need to be processed to make it thinner it needs sectioning in these cases. The choice of preparation technique is ultimately determined by the nature of the sample and the resolution required for the intended biological insight, but common methods include negative staining, plastic embedding, sectioning and vitrification.

EM grids are traditionally 3.05 millimetres across and made from a mesh of metals such as copper, gold, nickel, molybdenum or rhodium.

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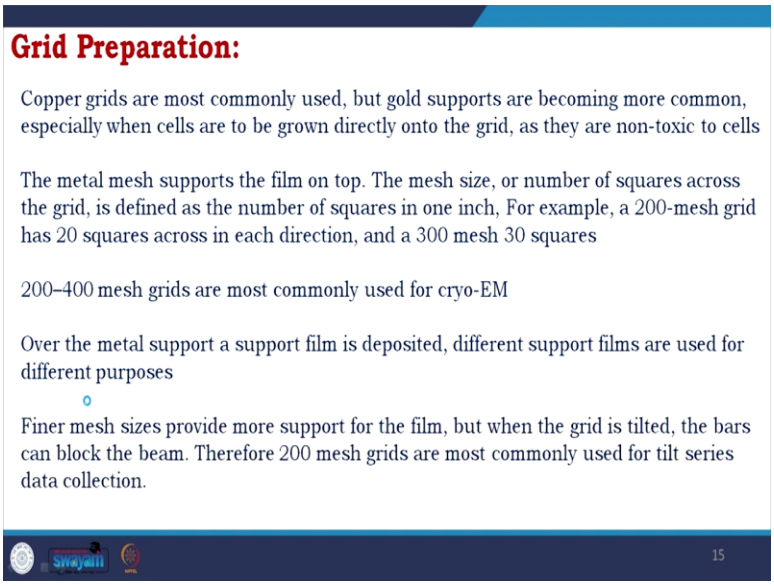

The most commonly used grids are copper grids, but gold supports are becoming more common, especially when cells are grown directly onto the grid; the gold supports are non-toxic to the cells. The metal mist supports the film on top. The mesh size or number of squares across the grid is defined as the number of squares in one inch. For example, a 200 mesh grid has 20 squares across each direction and a 300 mesh 30 squares. 200 to 400 mesh grids are most commonly used for cryo-EM. Over the metal support, a support film is deposited. Different support films are used for different purposes. Finer mesh sizes provide more support for the film, but the bars can block the beam when the grid is tilted. Therefore 200 mesh grids are most commonly used for tilt series data collection.

So, any mesh grid could be used, but for tilting experiments, you have optimization, and there 200 mesh grids work the best. So, this is about grid preparation.

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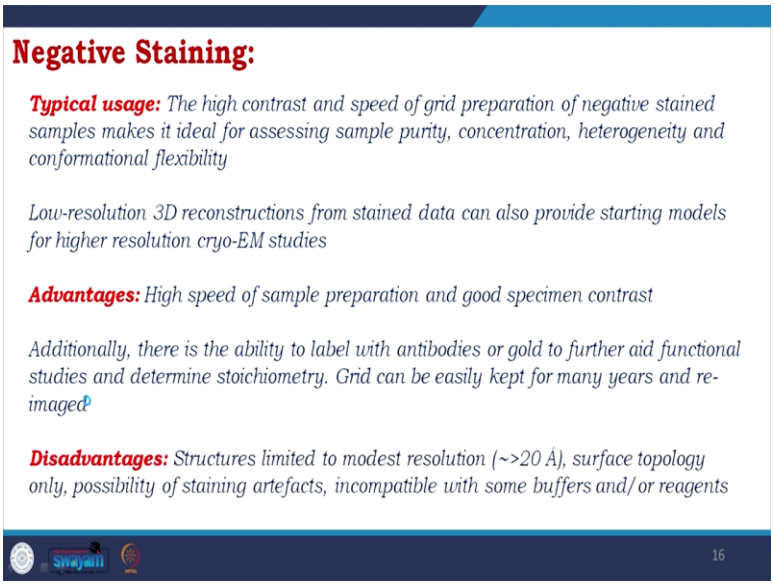

We come to negative straining summarizing it. Typical usage of negative staining and high contrast and speed of grid preparation of negative strain samples make it ideal for assessing sample purity, concentration, heterogeneity, and conformational flexibility. So, we could quickly see the standard or assess the sample quality by doing negative staining. Low-resolution 3D constructions from strain data can also provide starting model for higher resolution cryo-EM studies.

Advantages: high speed of sample preparation and good specimen contrast. Additionally, there is the ability to label with antibodies or gold to further aid. Functional studies and determine stoichiometry. Grids can be easily kept for many years and re-imaged. Disadvantages structures limited to modest resolution around 20-angstrom surface topology only possibility of staining artefacts incompatible with some buffers or reagents.

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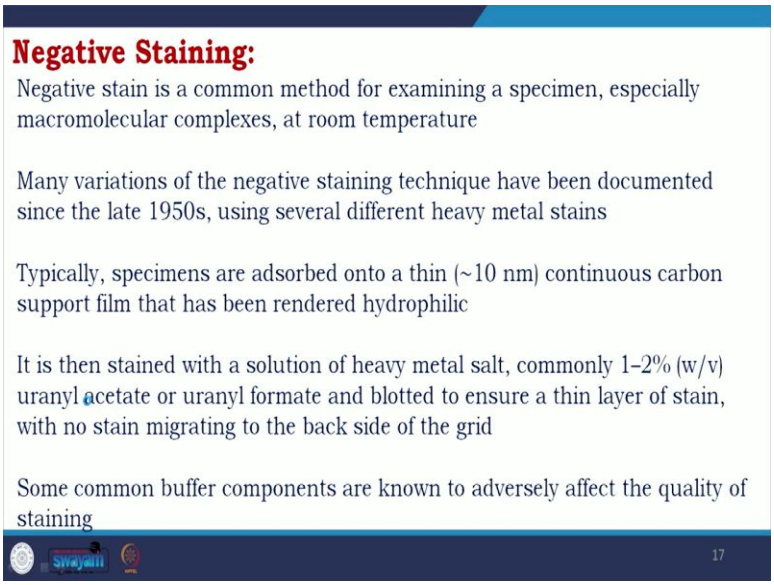

A negative stain is a common method for examining a specimen, especially a macromolecular complex at room temperature. It is a common method; it is the most common method. Many variations of the negative straining technique have been documented since the late 1950s using several different heavy metal stents. Typically specimens are adsorbed onto a thin 10-nanometer continuous carbon support film that has been rendered hydrophilic.

It is then stained with a solution of heavy metal salt, commonly 1 to 2% weight by volume uranyl acetate or uranyl format and blotted to ensure a thin layer of stain with no stain migrating to the backside of the grid. So, you have the grid the staining, and you transfer it to see how it works. Some common buffer components are known to affect the staining quality adversely.

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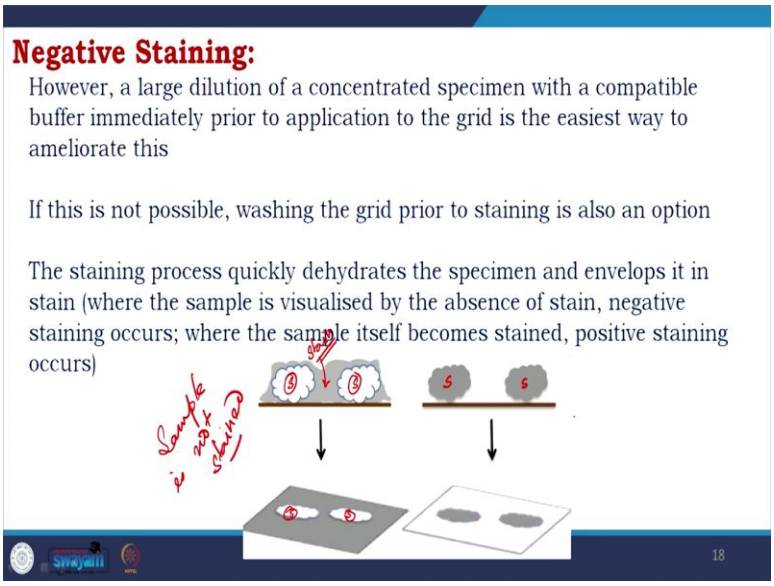

However, a large dilution of a concentrated specimen with a compatible buffer immediately prior to application to the grid is the easiest way to ameliorate this. If this is not possible, washing the grid prior to staining is also an option, like when you always try to set it up so that you have a grid you fix, you have the stain, and it does not go in the other side but, how you could do that? One way is dilution, proper dilution, but wash it properly if possible.

The staining process quickly dehydrates the specimen and envelops it in stain, where the absence of strain visualizes the sample negative staining occurs, but when the sample itself becomes strain positive, staining occurs. So, the sample is unstained negatively stained. So, here the sample is not stained; here, the sample is strained. So, this is positive straining.

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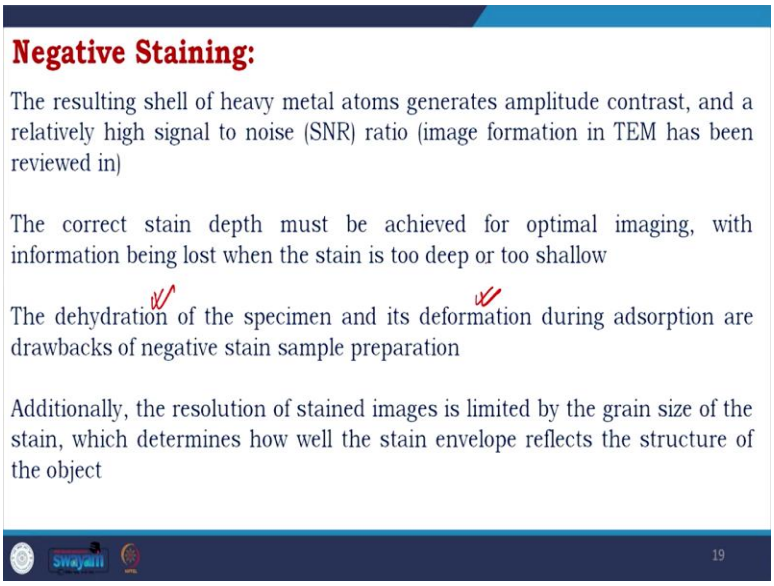

The resulting shell of heavy metal atoms generates amplitude contrast and a relatively high signal to noise ratio, which you see in the image. The correct stain depth must be achieved for optimal imaging, with information being lost when the stain is too deep or too shallow. The dehydration of the specimen and its deformation during adsorption are drawbacks of negative strain sample preparation.

So, one is the possibility of dehydration and deformation. Additionally, the resolution of the stained image is limited by the grain size of the stain, which determines how well the strain envelope reflects the structure of the object.

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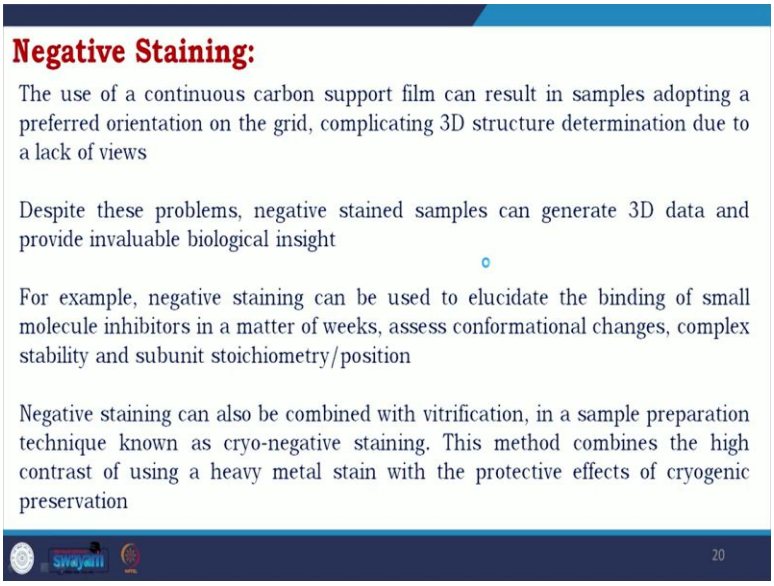

The use of a continuous carbon support film can result in samples adopting a preferred orientation on the grid, complicating 3D structure determination due to a lack of views. A negative stain sample can generate three data and provide valuable biological insight despite these problems. For example, negative straining can elucidate the binding of small molecule inhibitors in a matter of weeks assess conformational changes, complex stability and subunit stoichiometry or position.

So you get information about biological interactions. Negative staining can also be combined with vitrification. We will discuss that in a sample preparation technique known as cryo negative staining, discussed in the general principle. This method combines the high contrast of using a heavy metal strain with the protective effect of cryogenic preservation. So you use a heavy metal stain, but since the temperature is low. So, the effect is protected.

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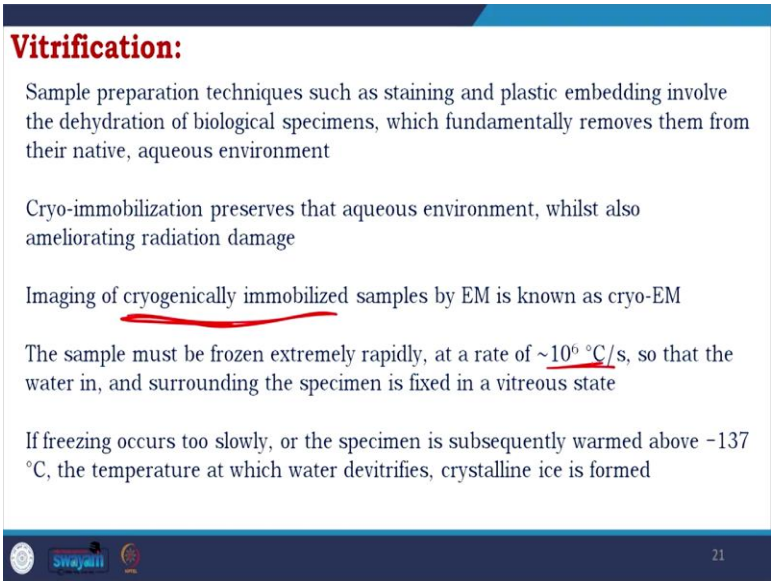

Vitrification is one of the critical concepts where cryo-electron microscopy works; This is sample preparation techniques such as staining and plastic embedding. Involve the dehydration of biological specimens, which fundamentally removes them from their native aqueous environment, which we do not want. The standard sample preparation procedure where the preparation we get the non-native condition.

So, cryo-immobilization preserves that aqueous environment whilst also ameliorating radiation damage. The higher mass electrons are doing radiation damage, especially in the biological sample; the radiation damage is critical. So, with the development of cryo-immobilization, the aqueous environment is preserved, the native solution environment we want for the reaction or the information to get, and the radiation damage reduction.

Imaging of cryogenically immobilized samples by electron microscopy is known as cryo-EM. As I told you, this is the one that gives the identity of cryo-EM. The identity of cryo-EM to work with the biological samples Even though TEM and SEM have been used for a long time, these cannot be used in biological samples because they lead to radiation damage.
Cryogenic immobilization makes cryo-EM today's cryo-EM. In the next class, when I am talking about the high resolution we are getting today, and how to achieve it, one of the main reasons is that the sample must be frozen extremely rapidly at a rate of 10 to the power 6 degrees centigrade per second so, that the water inside and surrounding the specimen is fixed in a vitreous state that is called vitrification.

If freezing occurs too slowly or the specimen is subsequently warmed above minus 137 degrees centigrade, the temperature at which water de-vitrifies crystalline ice is formed.

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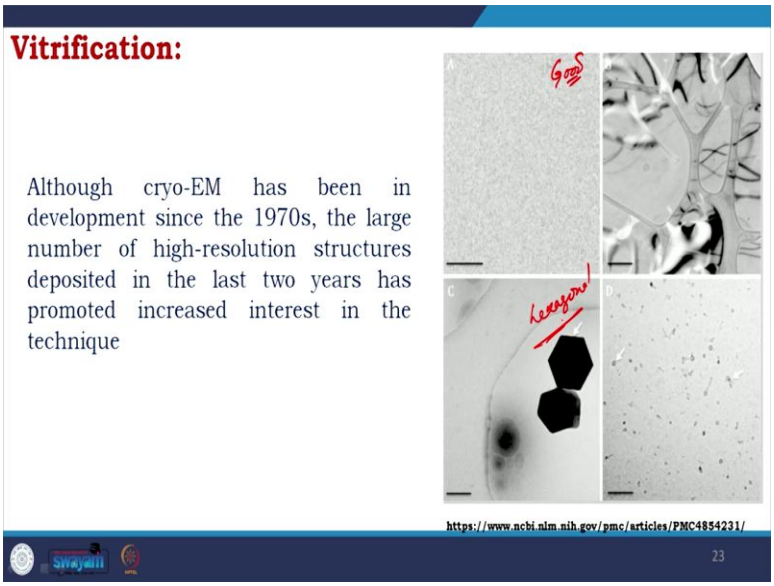

According to the experienced users, warming the sample by more than minus 160 degrees centigrade can greatly give poor quality ice, affecting the experimental data. Ice contamination compromises the structural integrity of the specimen as crystals withdraw water molecules from the hydration cell of the specimen or the specimen itself. The formation of crystalline ice also degrades image quality as they have different electrons.

So, the ice becomes crystals; So, they start diffracting the electrons again, creating another complication. Contamination can also occur during the freezing process, such as hexagonal ice, which can be reduced by working in humidity control environments and minimizing ice contamination in the liquid nitrogen. It could come from liquid nitrogen, which is used for freezing.

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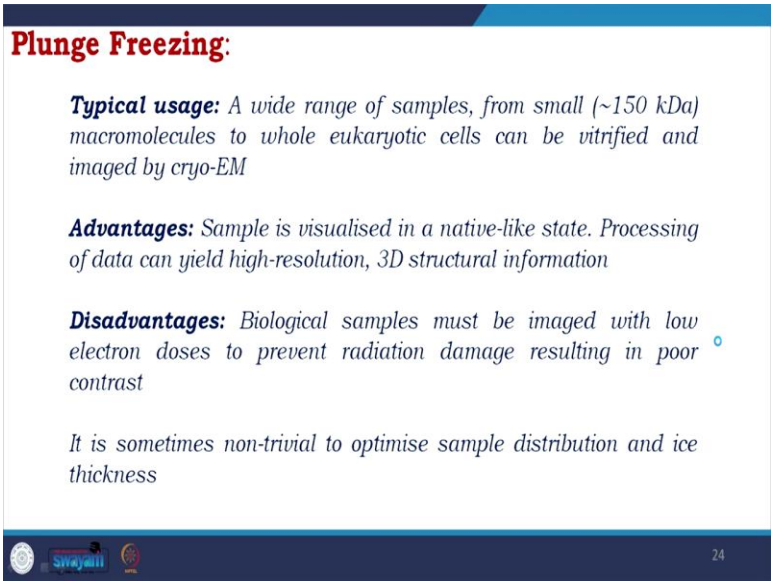

Although cryo-EM has been in development since 1970, many high-resolution structure deposits have promoted increased interest in the technique in the last two years. As you see here, good vitrification happened here; you can see the presence of crystals. Here you can see the hexagonal crystal impurity or contaminant.

Here you see the dots; these form in non-homogeneous conditions. So we talked about sample preparation, negative steering, and vitrification. How could we bring the vitrification? One of the methods is plunge freezing. A wide range of samples from small 150 kilodalton macromolecules

to whole eukaryotic cells can be vitrified and imaged by crime using the plunge freezing technique.

The sample is visualized in a native-like state; data processing can yield high resolution 3D structural information obtained with ease and comfort. Disadvantages: biological samples must be imaged with low electron doses to prevent radiation damage resulting in poor contrast. It is sometimes non-trivial to optimize sample distribution and ice thickness.

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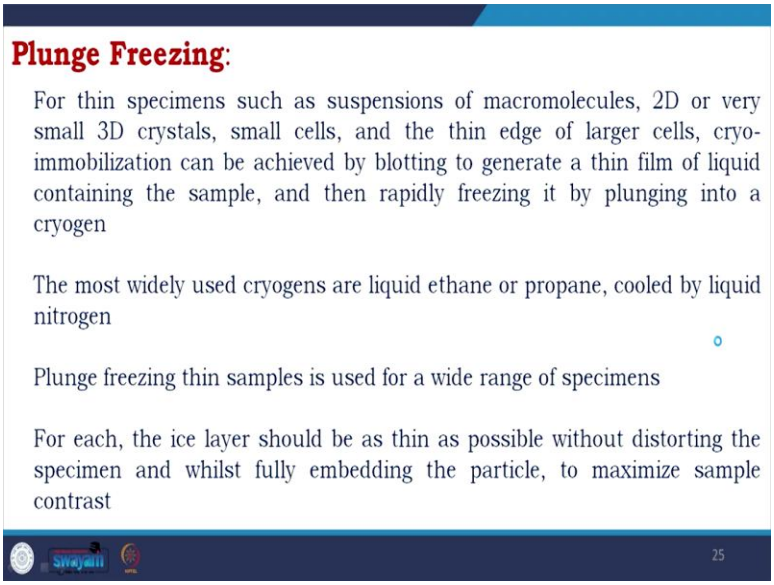

So, when you have thin specimens such as suspension of macromolecules, 2D or very small 3D crystals, small cells, and the thin edge of the larger cells, cryo immobilization can be achieved by blotting to generate a thin film of liquid containing the sample and then rapidly freezing it by plunging into a cryogen. So, you have the thin film you do the blotting and freeze it rapidly; the most widely used cryogens are liquid ethane or propane cooled by liquid nitrogen.

Plunge freezing thin sample is used for a wide range of specimens. For each, the ice layer should be as thin as possible without distorting the specimen and whilst fully embedding the particle to maximize the sample contrast.

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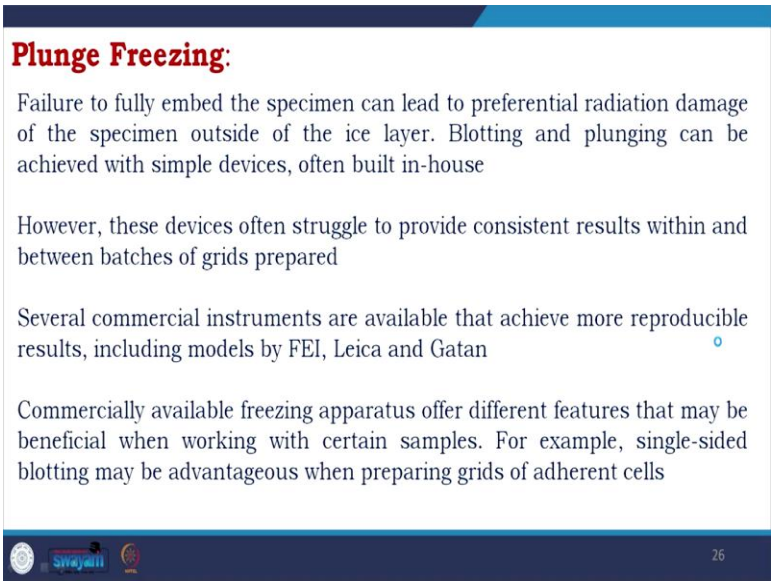

Failure to fully embed the specimen can lead to preferential radiation damage because if you could not embed it, there would be radiation damage outside the ice layer. Blotting and plunging can be achieved with simple devices often built-in-house for your requirement. However, these devices often struggle to provide consistent results within and between grids prepared.

Several commercial instruments that achieve more reproducible results including models by FEI, Leica, and Gatan. Commercially available freezing apparatus offer different features that may be beneficial when working with certain samples. For example, single-sided blotting may be advantageous when preparing grids of adherent cells because, otherwise, addition property would be a problem.

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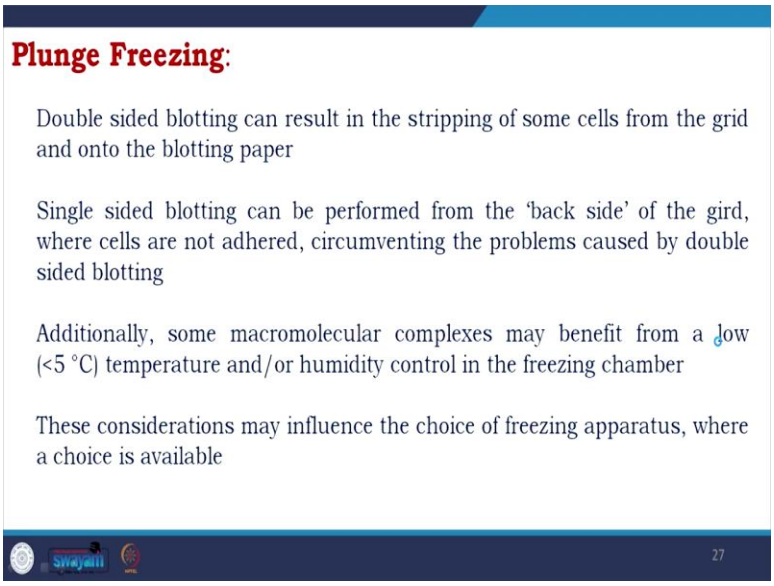

Double-sided blotting can strip some cells from the grid and onto the blotting paper. Single-sided blotting can be performed from the backside of the grid where cells are not added, circumventing the problem caused by double-sided blotting. Some macromolecular complexes may benefit from a low around 5-degree centigrade temperature or humidity control in the freezing chamber.

These considerations may influence the choice of freezing apparatus whether the choice is available to you or not.

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So, there are mainly three popular commercially available front freezing devices Leica EM GP, Electron Microscope, Gatan cryo plunge 3 and FEI vitrobot. So, in Leica, the humidity control in

the chamber is present it is also present in FEI vitrobot, but in Gatan cryo plunge 3, it is not present. The chamber always set at 98% humidity temperature control in the chamber is present in Leica and FEI vitrobot, but it is not present in Gatan; it operates at ambient temperature.

Automatic control of ethane temperature is present in Leica and present in Gatan but absent in Vitrobot. Blotting modality is single-sided as we have seen that single-sided are advantageous in many cases. Gatan is single and double-sided depending on the requirement and vitrobot double-sided. Some speciality for Leica optional stereo microscope to monitor blotting is an additional facility. Gatan removable humidity chamber as the chamber is set at 98 degrees if required it could be removed. For vitrobot, currently, the most common plunge freezing device is mostly used.

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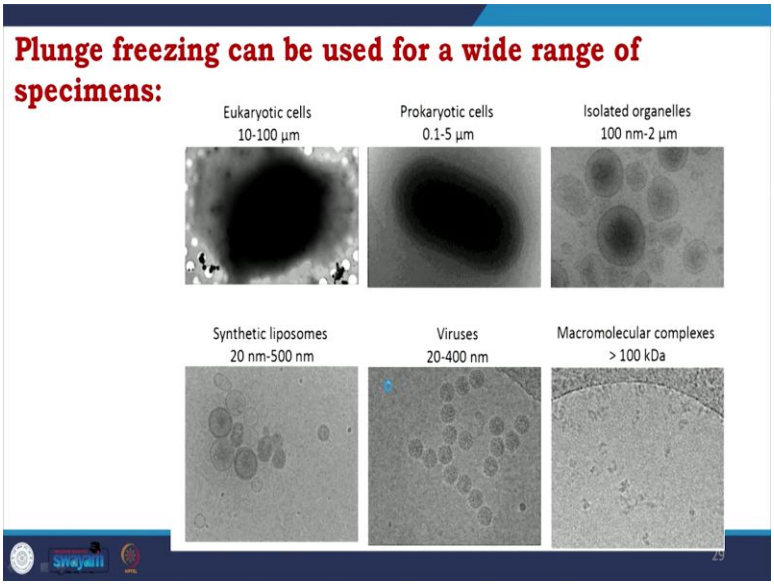

So, these are different scenarios where plunge freezing is used for eukaryotic cells, prokaryotic cells isolated organelles, synthetic liposomes, viruses, macromolecular complexes more than a hundred kilodalton you see the image after plunge freezing. We talked about plunge freezing; plunge freezing is mainly applicable to thin samples. Whenever your sample is thick, you need high-pressure freezing.

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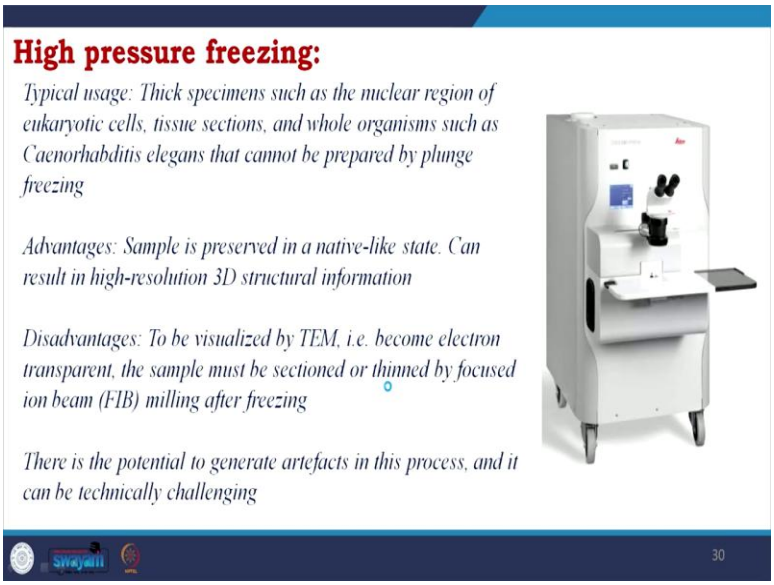

Thick specimens such as the nuclear region of eukaryotic cell tissue sections and whole organisms such as *C.elegans* that cannot be prepared by plunge freezing are going under high-pressure freezing. The advantage is that the sample is preserved in a native-like state, resulting in high resolution 3D structural information. The disadvantage is to be visualized by TEM that is become electron transparent. After freezing, the sample must be sectioned or thin by focus ion beam or FIB milling.

So, sectioning is required. There is the potential to generate artefacts in this process, and it can be technically challenging to make the sectioning after the freezing.

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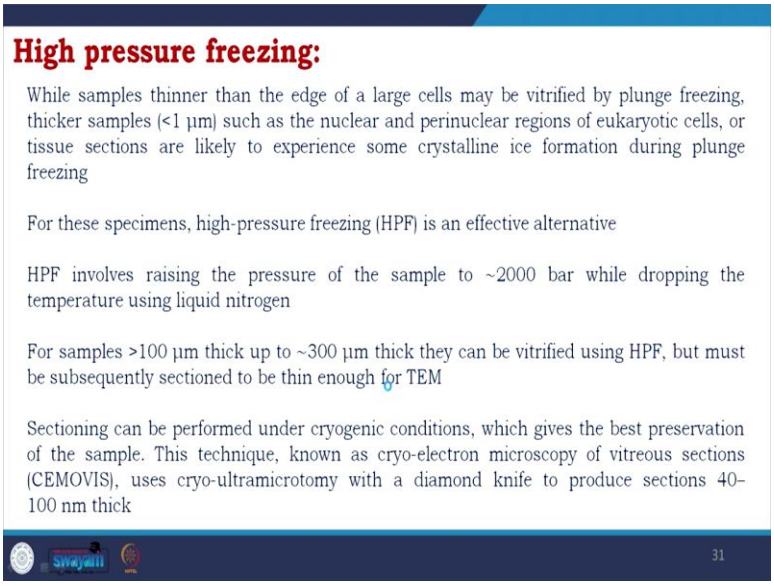

While samples thinner than the edge of a large cell may be vitrified by plunge freezing, thicker samples such as nuclear and fairy nuclear regions of eukaryotic cells or tissue sections the likely to experience some crystalline ice formation during plant freezing because of the thickness of the sample. High-pressure freezing HPF is an effective alternative for these specimens. High-pressure freezing or HPF involves raising the pressure of the sample to 2000 bar while dropping the temperature using liquid nitrogen.

So, the low temperature is achieved by making the pressure hugely high, around 2000 bar. For samples that are around or greater than 100 micrometres thick up to 300 micrometres thick, they can be vitrified using high-pressure freezing but must be subsequently sectioned to thin enough for transmission electron microscopy. Sectioning can be performed under cryogenic conditions, which gives the samples the best preservation.

This technique is, known as cryo-electron microscopy of vitreous section or CEMOVIS, uses cryo ultramicrotome with a diamond knife to produce sections 40 to 100 nanometer thick. So, a special system that uses cryo ultra microtomy using a diamond knife produces 40 to 100 nanometer thick sections.

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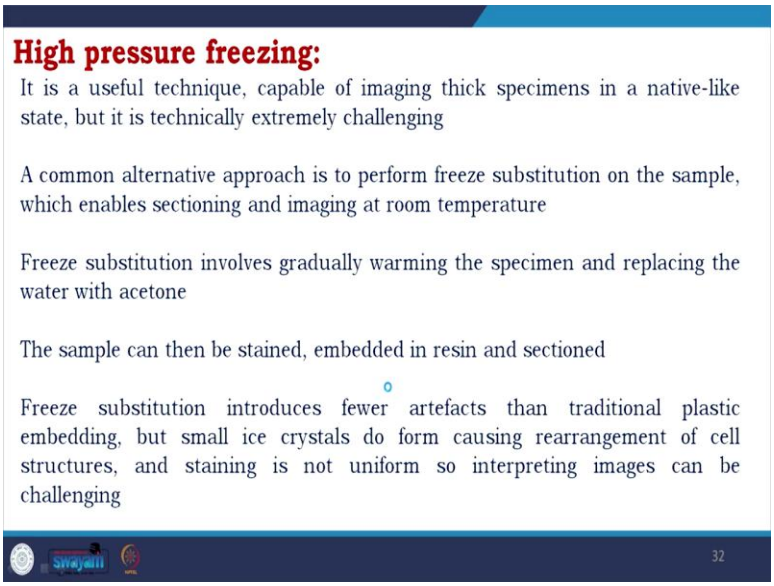

High-pressure freezing is a useful technique capable of imaging thick specimens in a native-like state, but it is technically extremely challenging. A common alternative approach is to perform

fridge substitution on the sample, enabling sectioning and imaging at room temperature. Freezing substitution involves gradually warming the specimen and replacing the water with acetone. The sample can then be stained, embedded in resin and sectioned.

Freeze substitution introduces fewer artefacts than traditional plastic embedding, but small ice crystals form, causing cell structure rearrangement and straining is not uniform. So, interpreting images can be very challenging.

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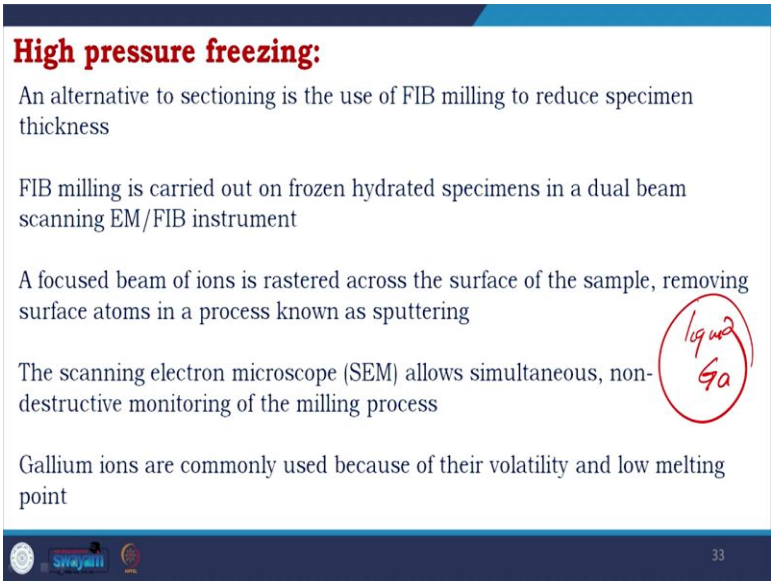

Moreover, in alternative to sectioning, FIB milling is also used to reduce specimen thickness; FIB milling is carried out on frozen-hydrated specimens in dual-beam scanning, electron microscopy and FIB instrument. A focused ion beam is rested across the sample's surface, removing the surface atom in a process known as sputtering. The scanning electron microscope allows simultaneous non-destructive monitoring of the milling process.

Gallium ions are commonly used because of their volatility and low melting point; if you remember, I talked about liquid gallium. Liquid gallium is used as the target in x-ray crystallography.

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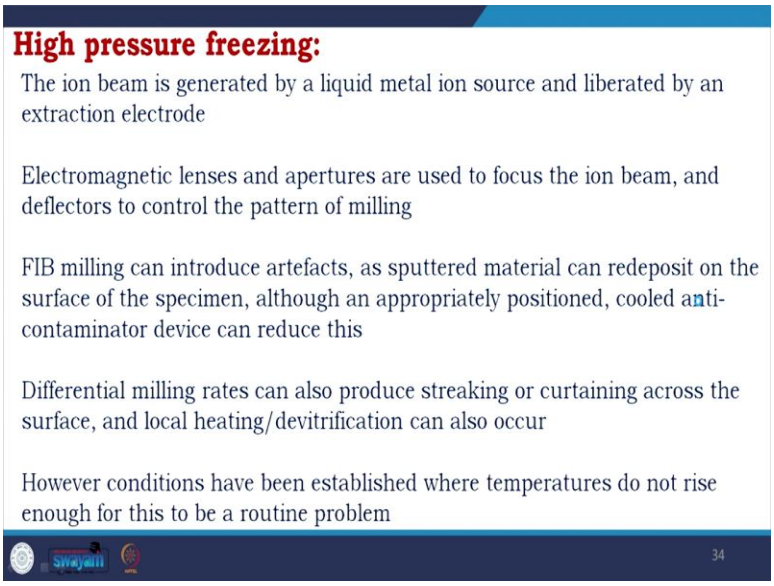

The iron beam is generated by a liquid metal ion source and liberated by an extraction electrode. Electromagnetic lenses and apertures are used to focus the ion beam and deflectors to control the pattern of milling. FIB milling can introduce artefacts as sputtered material can redeposit on the surface of the specimen, although an appropriately positioned cooled anti-contaminated device can reduce this effect.

Differential milling rates can also produce tricking or cutting across the surface, and local heating de-vitrification can also happen. However, conditions have been established where temperatures do not rise enough to be a routine problem.

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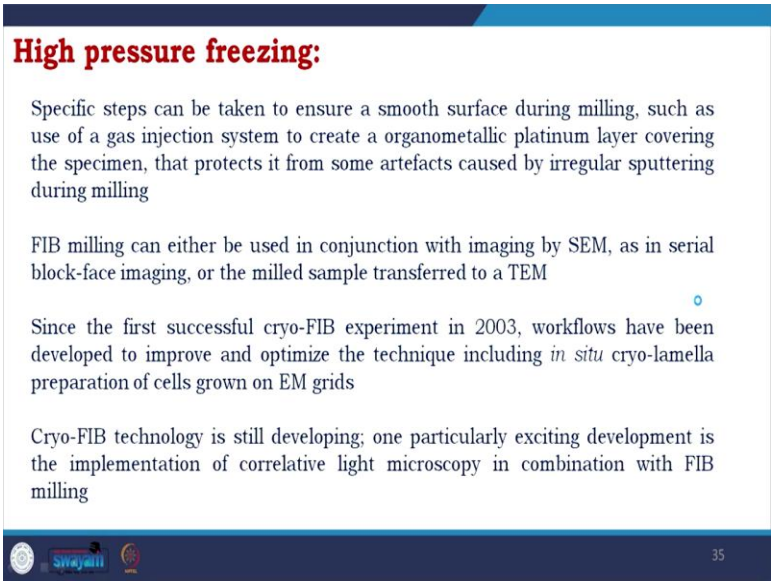

For ensuring a smooth surface during milling, specific steps can be taken, such as using a gas injection system to create an organometallic platinum layer covering the specimen that protects it from artefacts caused by irregular sputtering. FIB milling can either be used in conjunction with imaging by SEM as in serial block-face imaging or the milled sample transfer to the transmission electron microscopy.

Since the first successful cryo-FIB experiment in 2003, workflows have been developed to improve and optimize the technique including in situ cryo lamella preparation of cells grown on EM grids. Cryo-FIB technology is still developing; one fascinating development is the implementation of correlative light microscopy in combination with FIB milling.

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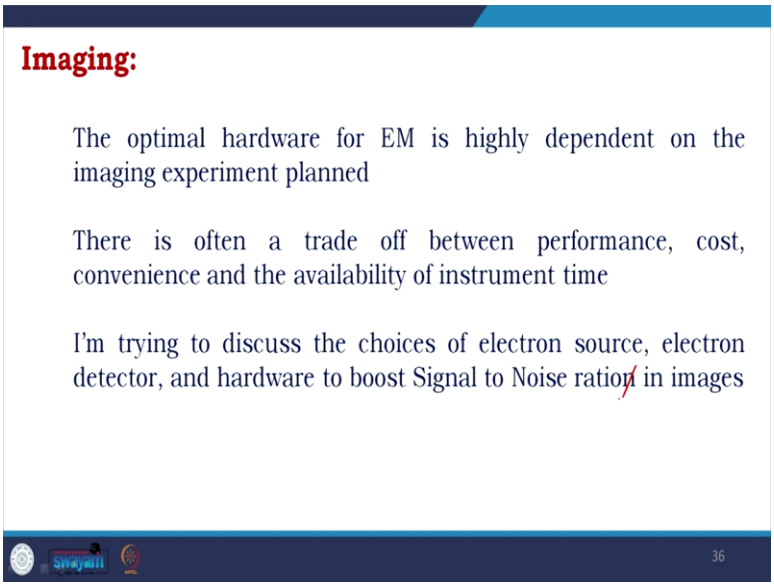

We will talk about special instrumentation in the next class; now I will talk about imaging. So we have the sample preparation, then we do the staining, then we go for freezing and imaging. The optimal hardware for an electron microscope depends on the planned imaging experiments. There is often a tradeoff between performance, cost, convenience and the availability of the instrument time because we all know that it is costly.

Here I am discussing the electron source, electron detector, and hardware choices to boost the signal to noise ratio in the images. What is the instrumentation, and what it is affecting?

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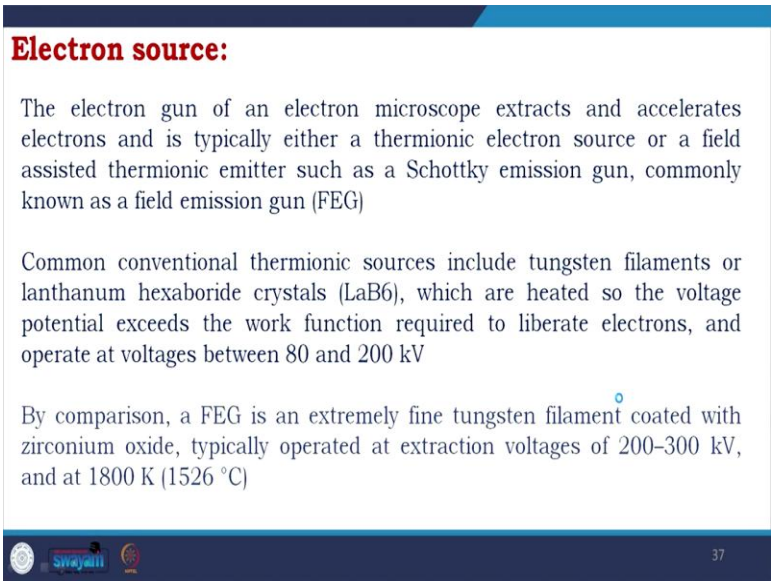

Electron source very commonly uses the electron gun or filth emission gun FEG. So, the electron gun of an electron microscope extracts and accelerates electrons and is typically either a thermionic electron source or a field assisted thermionic emitter such as the Schottky emission gun. They are commonly known as field emission guns or FEG. Common conventional thermionic sources include tungsten filaments or lanthanum hexaboride crystals LaB6, which we discussed earlier, which are heated.

So, the voltage potential exceeds the work function required to liberate the electrons and operate at a voltage between 80 to 200 kV. By comparison, a field emission gun is an excellent tungsten filament coated with zirconium oxide typically operated at extraction voltage of 200 to 300 kilovolt and at 1800 Kelvin, which means 1526 degrees centigrade.

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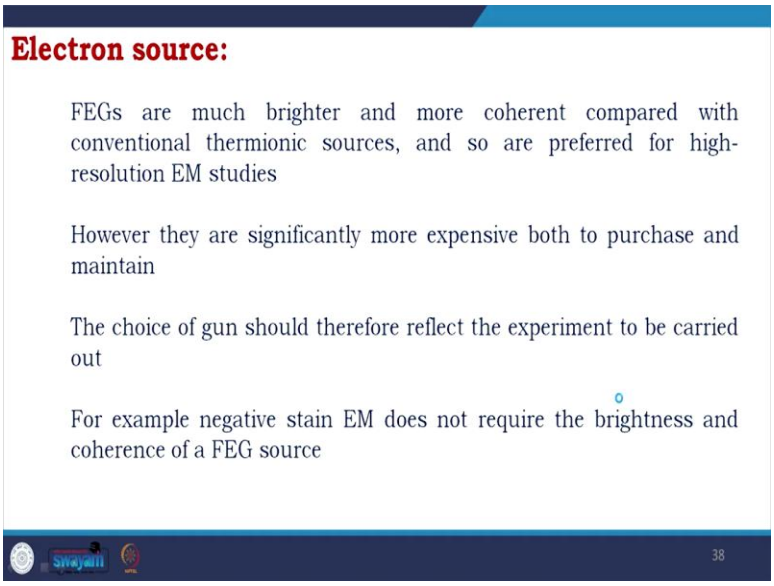

Field emission guns are much brighter and more coherent than conventional thermionic sources and are preferred for high-resolution EM studies. However, they are significantly more expensive both to purchase and maintain. The choice of guns should therefore reflect the experiment to be carried out. For example, negative strain electron microscopy does not require the brightness and coherence of a field emission gun source.

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Coming to electron detectors: So, we have the source now we have to detect the high energy electrons used in the electron microscopy imaging are recorded using a detector. Recording devices include photographic film charge-coupled device cameras or direct electron detectors. So, we have already discussed the photographic film the CCDs when discussing the x-ray crystallography detection and the same; but direct electron detectors are something new. Cryo-EM images of biological specimens are intrinsically noisy due to the low electron doses used to prevent specimen radiation damage. A perfect detector would add no noise, but all detectors actually do that in practice; The noise or the effect of noise expressed as the detective quantum efficiency or DQE of the detector the square of the output signal to noise ratio SNRO, which is over the input signal to noise ratio SNRI. So, CSNRO is the signal to noise ratio of the output signal to the SNRI is the input signal to noise.

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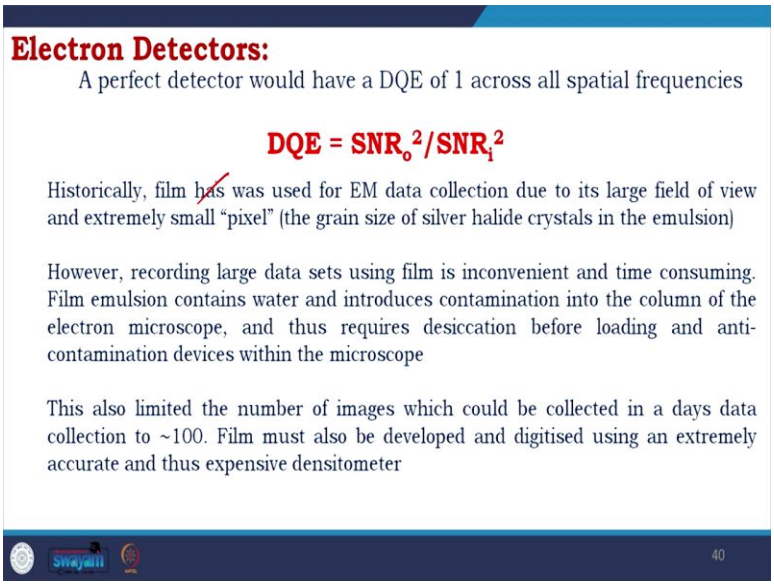

So, a perfect detector would have a DQE of 1 across all special frequencies. DQE is equal to the ratio of SNRO signal to noise of output square by input square. Historically film was used for electron microscopy data collection due to its large field up view and extremely small "pixel". However, recording large data set using films is inconvenient and time-consuming is the same thing we discussed in x-ray crystallography.

Film, emulsion, contents, water, and introduces contamination into the electron microscope column and thus requires desiccation before loading and anti-contamination devices within the microscope, which is not a user-friendly method. The use of film also limits the number of images collected in a day; data collection for around a hundred films must also be developed and digitized using an extremely accurate and thus expensive densitometer.

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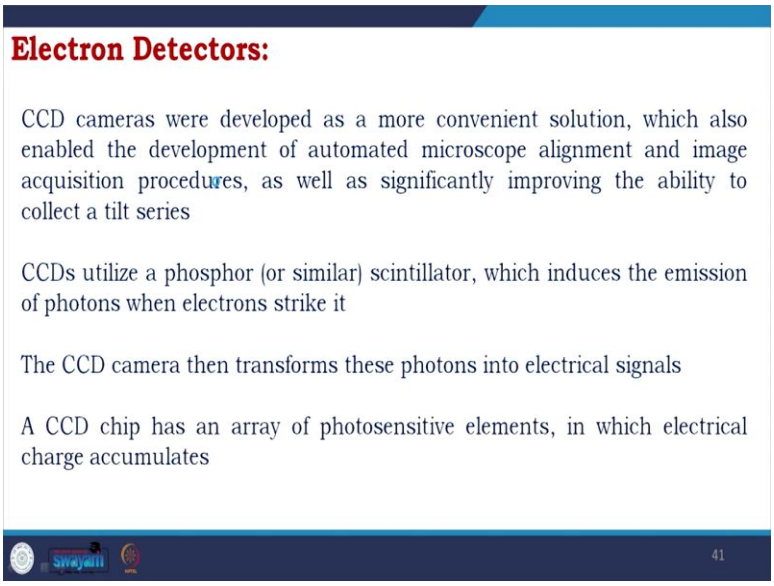

CCD cameras were developed as a more convenient solution; it enables the development of automated microscope alignment and image acquisition procedures and significantly improve the ability to collect a tilt series. CCDs utilize a phosphor or similar scintillator, which induces the emission of photons when electrons strike it. The CCD camera then transforms these photons into electrical signals.

A CCD chip has an array of photosensitive elements. I have shown you the details of how the electrical charge accumulates.

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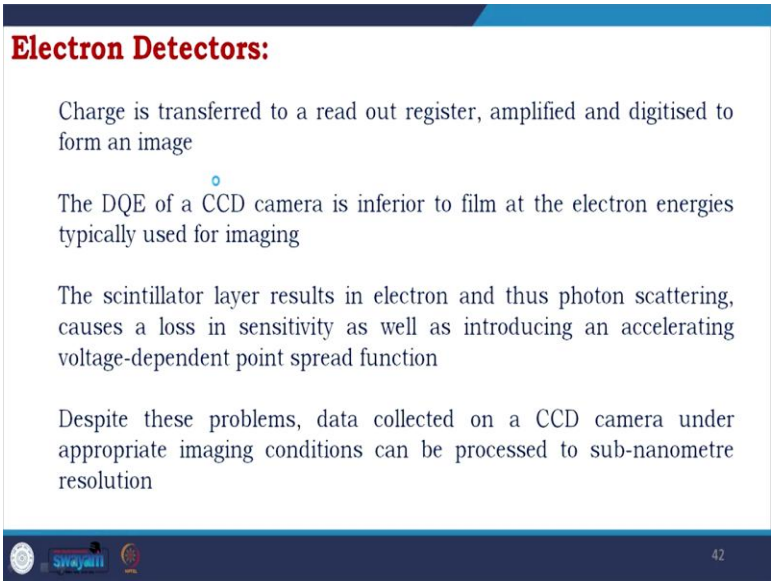

The charge is transferred to the readout register amplified and digitized to form an image. The DQE of a CCD camera is inferior to film with the electron energies typically used for imaging. The scintillator layer results in electron and thus photon scattering causes a loss in the sensitivity and introduces an accelerating voltage-dependent point spread function. Despite those problems, the data collected on a CCD camera under appropriate imaging conditions can be processed to sub-nanometer resolution. So, that is an advantage.

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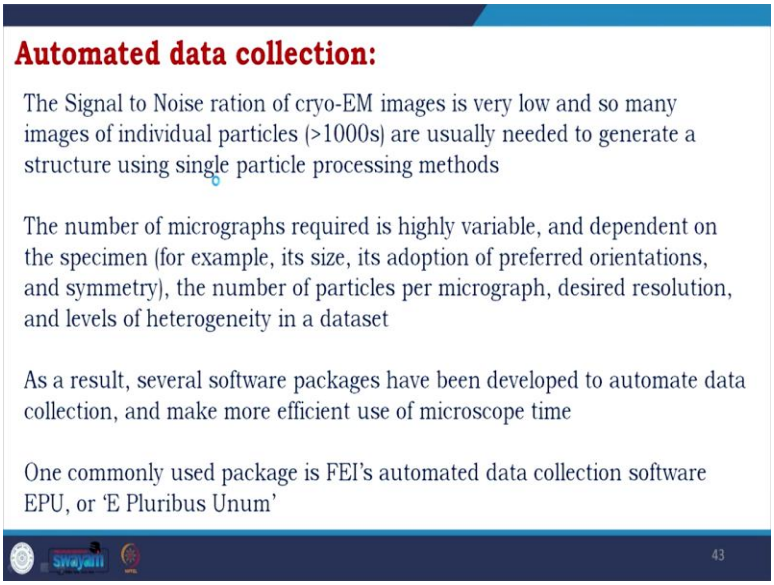

Coming to our last topic, automated data collection, the signal to noise ratio of cryo-electron microscopy images is very low. So, many images of individual particles greater than a thousand are usually needed to generate the structure using the single-particle processing method. So, more images you could collect better resolution you will get. The number of micrographs required is highly variable and depends on the specimen, such as its size and adoption of preferred orientation and symmetry.

The number of particles per micrograph desired resolution and levels of heterogeneity is all you need in the data set. As a result, several software packages have been developed to automate data collection and make more efficient use of microscope time. One commonly used package is FEI's automated data collection software EPU or E Pluribus Unum.

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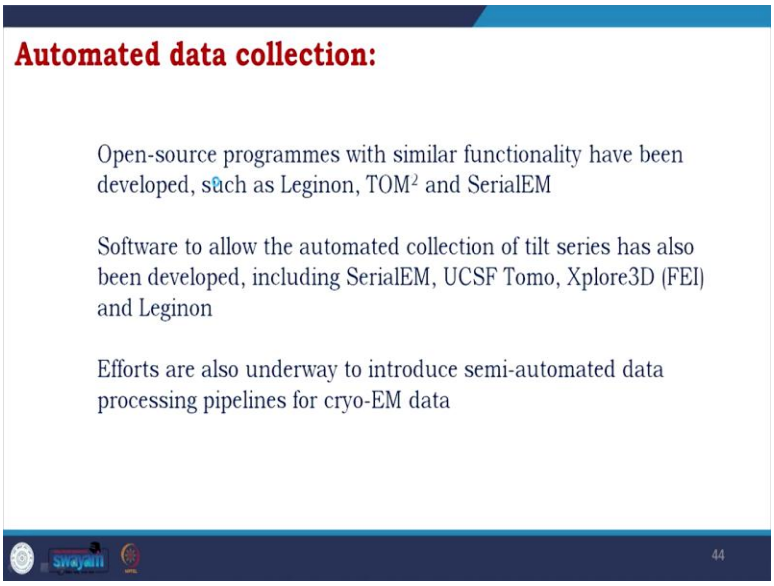

Open-source programs are also available with similar functionality, such as Leginon, TOM 2 and SerialEM. Software to allow the automated collection of tilt series, as we have discussed, the tilt series with angular data is essential. So, also been developed including serialEM, UCSF, Tomo Xplore 3D and Leginon. Efforts are also underway to introduce semi-automated data processing pipelines for the cryo-electron microscopy data. So, we have covered the basic functionalities and the process involved.

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So, if you see here you have a protein sample that contains the macromolecules in a grid; it is blotted, and the grid is put in the freezer and its plunge frozen, and now it is time for data collection you see that this is the cryo-electron microscope. The electrons are coming, and then they hit the sample, and the data is coming; you see the data is oriented in a different direction; the software makes the alignment similar oriented data within a similar position.

And then all those similar data is coming together, and your 3D model is ready; this is the 3D model you prepare for a macromolecule. So, in cryo-electron microscopy, you have electron microscopy, the first one to the proper grids, vitrification, different freezing methods, data collection where a lot of automation is going on, and last but not least, data analysis.

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We will talk about a few of these sections in more detail and discuss the history of how cryo-EM is proceeding. Moreover, in the next class, we will talk about data analysis. Thank you very much again; as I say every time, please keep listening and asking questions. Thank you very much for listening; thank you.