Analytical Technologies in Biotechnology
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Module - 1
Microscopy
Lecture - 7
Transmission electron microscopy continued and scanning electron microscopy

So, we were discussing in the last lecture about transmission electron microscopy. If you could recall, we were discussing about the specimen preparation techniques. We have discussed about one of the methods, where we it involved fixation, embedding, and sectioning, and finally staining to view the samples in electron microscopy.

Now, other than that conventional methods there are other methods of contrast creation for electron microscopy. Let us, little bit discuss about some of those methods. Now, one is negative staining. Negative staining is used for observation of samples containing small particulate material such as sub cellular fractions viruses and some macro and protein complexes. Here, the specimen is applied to a grid covered with a support film and is surrounded by an electron dense substance. These particles in the sample appear electron lucent. The stains used most frequently are 1 to 2 percent solutions of uranyl acetate or phosphor tungstic acid ammonium molybdate methyl amene tungstate and uranyl formate.

Now, the sample suspension which is 3 to 5 micro litre is placed on the grid. These are carbon coated grids. They are made up of hydrophilic, made hydrophilic by glow discharge. Shortly before applying the sample, what you have is that particles adhere to the film. The edge of the grid is then tested with a piece of paper to soak reminder of the suspension. By capillary action 3 to 5 micro litre of negative stain is applied for some time. After drawing of the stain solution grid, it is dried and it is immediately observed in transmission electron transmission microscopy.

Let me show you this on the screen and give you a feeling about how this whole thing happens. The negative staining like I said, are not really staining the specimen or the particulate matter which could be anything. Rather, you are surrounding it with the stain. The stain gets into the crevices and crafts which are present in here. So what you have is,

you have like electron rich area surrounding but the object or specimen is electron translucent.

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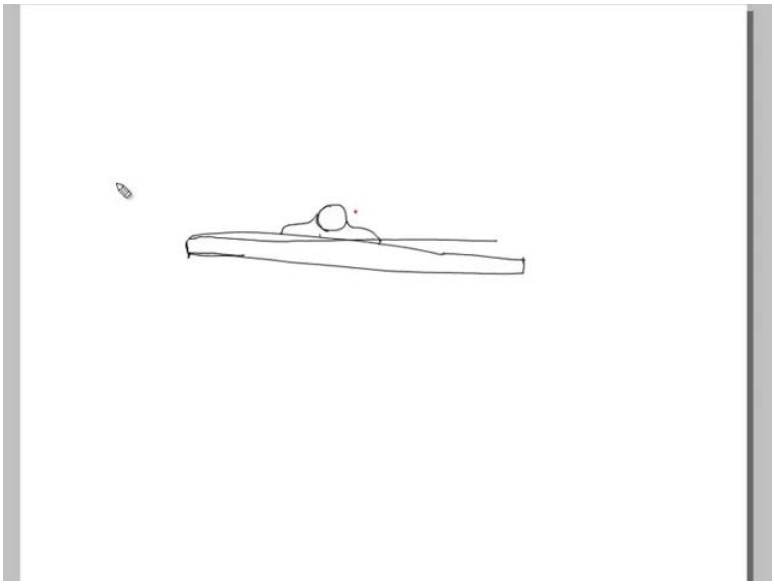

So, what you have is essentially, you will see here that if you have a particular area what you have is. If a material is put in here, then this material is surrounded by a particular stain. Now, this stain will get into the surface crevices and the other imperfections and that will give you a contrast. You will finally get an image and this could be dried off later on. The extra thing could be dried off so this one method is very good. It gives a good contrast and could provide you with images for certain specimens. It is not applicable for a lot of different applications. It is used for observations of samples containing particulate materials or sub cellular fractions.

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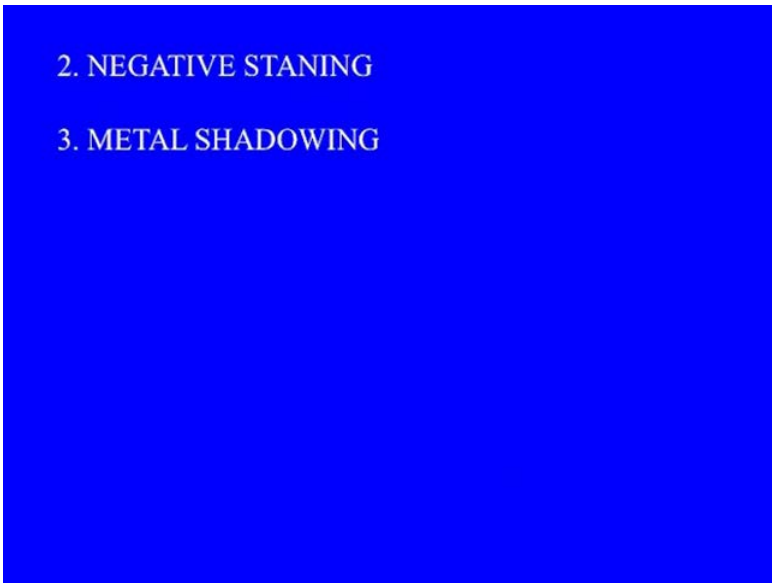

Now, another method is metal shadowing. Now, metal shadowing is another widely used technique to increase the contrast of a certain specimen by depositing thin coat of metal onto the surface. It cast shadows. What does that mean? Casting shadows, we will just try to make you understand this. This whole thing is performed in a vacuum evaporator containing one or more pairs of electrodes. It is connected to a dual pumping system which is rotary and oil diffusion pumps.

The metal to be evaporated is held between a pair of electrodes and heated by an electric current. This whole thing is done at an angle of 45 degrees or less. Your metal is placed around 45 degree or less to the sample section. The metal which could be platinum or sometimes tungsten together with carbon is deposited on surfaces. These are facing the filament that is which are towards the filament side.

While the opposite surfaces in specimen and grid will remain unquoted and incapable of scattering electrons. What you have is the sides which are quoted will scatter electrons and will not be seen in the florescent screen. The sides which are not quoted that are shadows will be passed electrons, will be passed from there and the florescent screen will be lighted up there.

Now, the areas in shadow appear bright on viewing screen. While, the metal coated areas will appear dark. This technique provides an excellent contrast for isolated materials and produces a 3 dimensional effect. In many metal shadowing technique there is another

technique which could be used which is rotary metal shadowing. In this rotary metal shadowing a thin film of metal is deposited on a specimen at very low angles. If you could see in metal shadowing it was 45 degree or less.

Here, 8 to 10 degree, at 8 or 10 degree. The metal is deposited while the sample is being rotated. The metal atoms deposit on structures projecting above the specimen surface. They will be quoted so molecular size shapes and interactions can be determined by use of this technique. Purified samples of nucleic acids proteins, protein interacting with nucleic acids etcetera can be seen in here on appropriate substrate.

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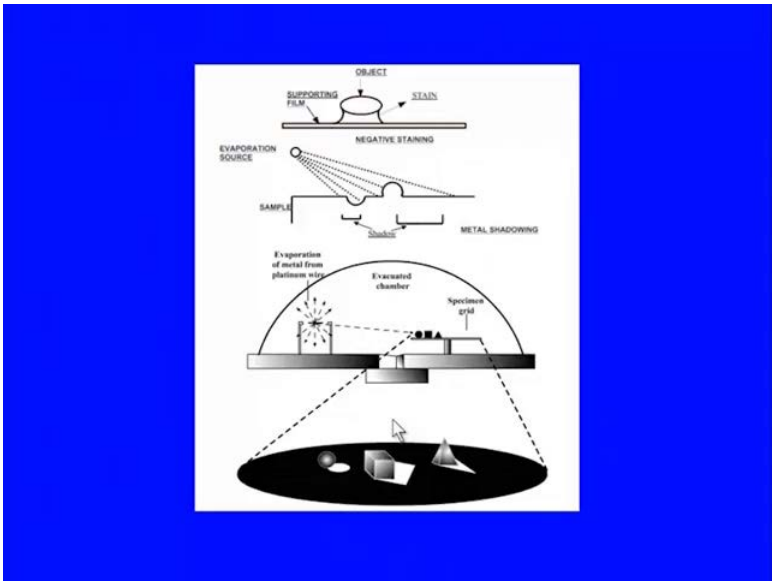

So, let us see how these figures, if you see this is for negative staining. What I have already showed to you is that the object is stained. There is a supporting film for metal shadowing which I was trying to explain. It is being done in an evacuated chamber and what is essentially done is that you have a metal which is being heated and evaporated. There is a sample with different ups and downs or different surfaces and structures.

When you are depositing this metal at an angle then only the exposed parts or the parts which are facing, which are facing the metal will be coated. The rest of the parts, you can see are not coated. These will be bright and seen on the screen. These will be seen in the dark on the screen. You can see here, the arrangement as such that the filament is hold between or held between two electrodes. It is at an angle and you will get a 3 dimensional effect of the images in rotary metal shadowing. This angle will be 8 to 10

degrees. Only the surface things which are projected outside will be quoted in here. That particular outer surface view could be seen.

So, this was another technique which was metal shadowing. We have seen negative staining and metal shadowing. These are the other two techniques which could be utilised for creating contrast. Other than that, there are cryotechniques. Cryotechniques mean, which are done in very cold conditions. Here, specimen preparation procedures are employing ultra cold conditions or temperatures are used. This could be liquid nitrogen to liquid helium temperature that is minus 196 degrees celsius to minus 271 degrees celsius.

In place of chemical fixation now, if you remember in chemical fixation you are doing chemical fixation, dehydration fixation and all those things which are not done in here directly. The main goal is to retain the native structure and properties of specimen. So let us see certain steps of cryotechniques. One is cryofixation, now in cryofixation you have to rapidly freeze the sample to preserve biological details. It is actually a very critical aspect. You would like to avoid the formation of ice crystals during freezing. If ice crystals forms they can disrupt cellular membrane and other structures. So freezing rates are very high and you avoid formation of crystals.

Cryogenic liquids such as liquid propane ethane or Freon have large differences in freezing and boiling points. These can be used here. The specimen needs to be plunged rapidly and deeply into a cryogen. One way is to use slam freezing devices. The slam freezing devices employs a solenoid driven plunger to which sample is attached. It is rapidly slammed against a polished copper surface cooled to liquid nitrogen or liquid helium temperature. So slam freezing is one way to freeze rapidly the sample.

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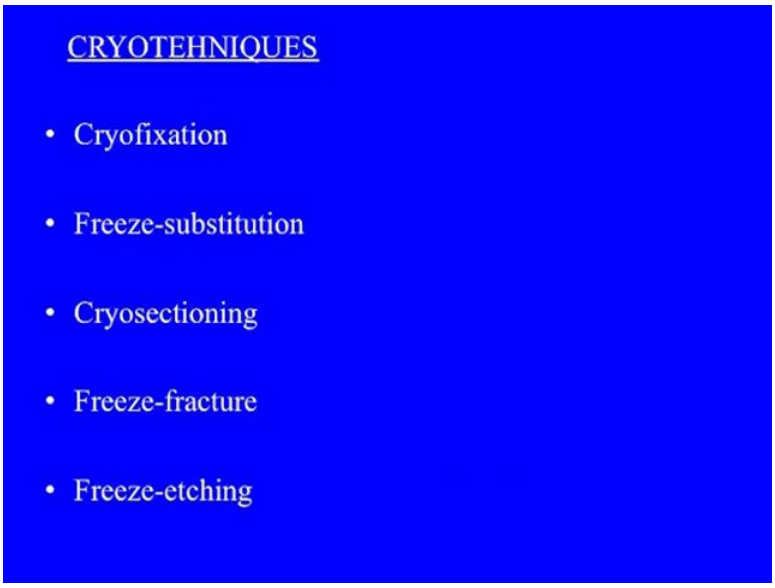

Now, freeze-substitution is done where frozen samples are placed in acetone or methanol at minus 85 degrees Celsius for several days. What it does it slowly brings it, dehydrates the sample by substituting frozen water by a particular sample like acetone or methanol. Then again fixatives like osmium tetroxide butaldehyde or paraformaldehyde can be used for fixation staining solutions like urinyl acetate. It can be added to solvent and at room temperatures. Samples can be embedded in epoxy raisins or in low temperature raisins the sectioning is done in cryo conditions. It is called cryosectioning thin areas; thin sections of cryo fixed samples can be prepared using a cryo ultra microtome. You have to see that microtome. If it is placed in the cold conditions it is called cryo ultra microtome.

This was about about cryotechniques. One of the cryotechniques which is widely used is the freeze-fracture technique. This technique is very important and it provides unique view of cellular membranes and distribution of integral proteins in the membrane. The specimens here are usually fixed in butaldehyde and cryo protected with 20 to 30 percent of glycerol before freezing. What is done is like; if you are preparing a sample for freeze fracture the first thing you have to do is that you have to fix the specimen to preserve the internal details. This is done by fixatives which could be butaldehyde.

Also, you have to cryo protect so that crystal formation could be avoided by say 20 to 30 percent glycerol. Before freezing once cryo protected, then these samples are rapidly

frozen and then they are transferred to a special stage in a vacuum evaporator. This is the modified version which could include a fracturing device. Now, fracturing device is required to have seen the membranes. This could be fractured at the middle and there is a scalpel or raiser which is mounted in these devices.

The fractured plane as we call it, usually takes the path of least resistance. Through the frozen block sample that is whichever path will give the least resistance or impart. Least resistance that part of fracture this will be followed. Now, fracturing is usually done at minus 100 degree Celsius and the fractured surface is immediately shadowed with platinum and carbon at an angle of 45 degree. This creates a replica of surface. With the carbon alone at an angle of 90 degree is used to reinforce the replica. Now, a specimen and a replica are placed on the foam wire. The foam wire coated grid specimen grid then grid will be viewed in the electron beam. The variation in thickness of the metal in different parts of the replica produces necessary contrast in this image.

So, there is another addition like extension of this particular technique. It could be more informative by a simple step called freeze-etching. Now, in freeze-etching what is done is that the frozen fracture samples in cold chamber are exposed to a vacuum. This is done at an elevated temperature for a very small period of time. What happens is a layer of ice gets evaporated from the surface. Once the water has been removed the surface of the structure, it can be coated with heavy metal, as information is exposed in here due to the water evaporation.

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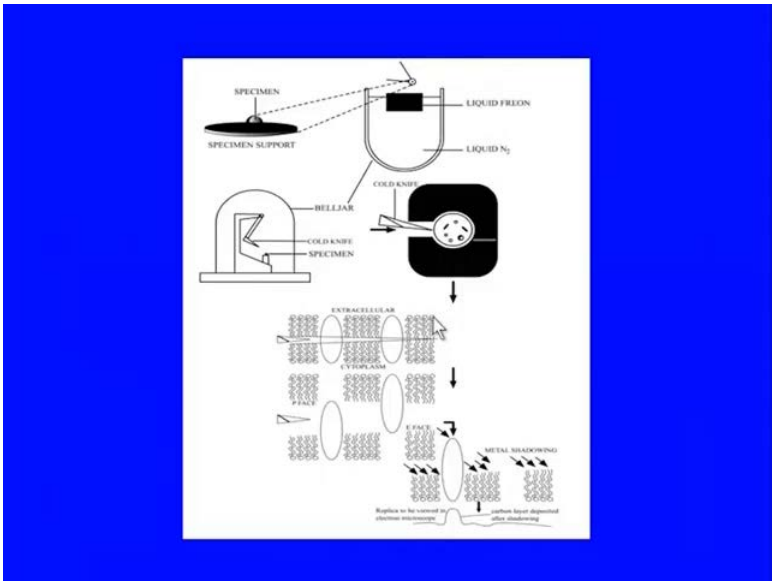

If you see in this figure, this figure clearly explains the freeze fracture technique. If you see here, the specimen is of a specimen support. There is a specimen, this specimen after a cryo protection through fixation and a cryo protection solvent like glycerol is used. This will be rapidly frozen and after rapidly freezing the sample will be put into this chamber which is cryo chamber or cold chamber. If you can see here, there is a scalpel or cold knife in here.

This cold knife is utilised to create the fracture. Like I said the fracture will take the path of least resistance. In the membranes, this is the path of least resistance it will take. It will be after the fracture you will see that you will get the two membrane or two mono layers. They are separated in the membrane by layers. But, integral proteins are in present in there. After this you will shadow it with the platinum or other metals and at 45 degree.

Again you will shadow it at 90 degree with carbon. What will happen is a replica is formed. You can see here a replica which is formed and this replica could be utilised to be used under the electron microscope. Rest of the things could be discarded. So since the replica has all different information as per the surface structure of the integral proteins. These are the other things that could be used for imaging in the electron microscope.

So, freeze-fracture technique is one of the very important techniques used in transmission electron microscope. This was about the scanning electron microscope. Let us move on to the sorry, this was about the transmission electron microscope. Now, let us move on to the

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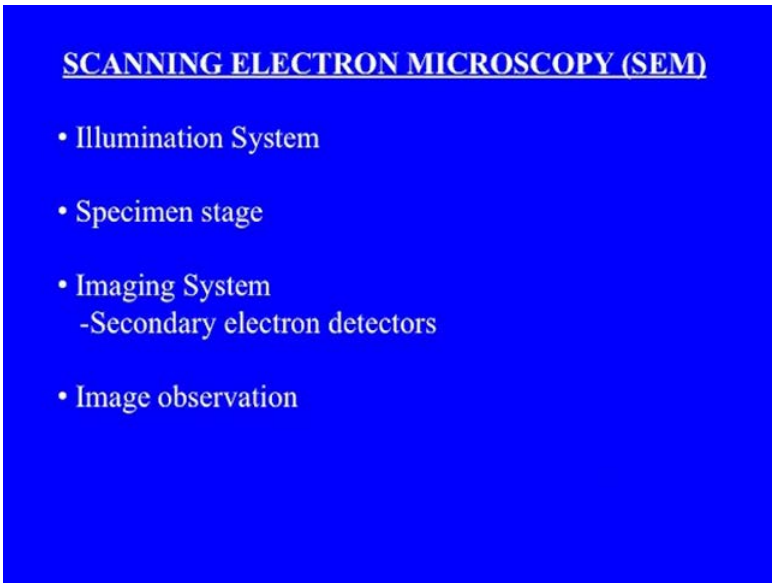

Next electron microscopy technique that is scanning electron microscopy. Electron microscopy in contrast to transmission electron microscopy will be utilised for surface viewing or surface details. Scanning electron microscopy is a type of technique that produces the image of a specimen by scanning it. It is done with a focussed beam of electrons across a rectangular area of the specimen or in a rester scan pattern. The electron beam interacts with a specimen either emission of low energy or secondary electrons or back scattered high energy electrons. It will provide signals carrying information about the specimen surface topography and composition.

Now, SEM has provided the means to examine in great clarity and detail. The object ranges in size from a virus to an animal head. We have a variety of specimens which could be used in here. The construction and operation of SEM are very different from TEM that is transmission electron microscopy. Let us see how this is different from TEM. We will be seeing the optics of this later on, in a little while. Now illumination system here though basic things are same, but there are noticeable differences.

One is that the cathode is maintained at a negative potential difference to anode around 2 to 30 kilovolt rather than 10 to 100. That is one difference. Then there are 3 condenser lenses are used in SEM and the specimen is not placed. Rather a specimen is preplaced at the bottom rather than at in the middle of the column. The third condenser lens is used to reduce further the size of the beam or probe which is around 5 nanometre in diameter. As

this lens is used to focus beam on specimen and thus focus image it is often called as the objective lens. There is nothing like projector lens. Here, you can call it projector lens or objective lens.

Size of the beam spot or probe is a major determinant of resolution in the scanning electron microscope with small probe. The volumes of specimen with which the beam electrons beam of electrons interact to give rise to secondary electrons. It is smaller thus the signal for each individual picture element making up the final image originates from a smaller part of specimen. Also, defection coils are used to shift and tilt the beam during operation and alignment. Deflection coils in final condenser lens are used to restore the beam over the surface of the specimen. The size of the area is covered by a rester pattern. It is inversely proportional to the magnification of the image.

We will see how and likewise the specimen stage or specimen carrier. It is quite different from the transmission electron microscopy. In the stage of scanning electron microscope it is designed to accept a wide range of specimen sizes up to say 100 millimetres or more in diameter. So you have to have much versatile specimen stage as compared to transmission electron microscope.

This has a large motion which could may go up to 50 millimetre or more in x y direction. It can rotate a full 360 degree. The specimen stage can tilt up to 90 degree. In addition to changing the angle of viewing, tilting and rotating the specimen relative to electron detectors may help to enhance the signal. You can manipulate it in various ways. Other important parts, is that the stage can be raised or lowered.

That is you can call it is called z adjustment. You can lower or raise the stage to accommodate large or small specimens. The working distance or the focal length of final lens is varied in order to focus the electron beam on the surface of the specimen. Now, long working lens increase field depth but decrease resolution and signal strength so that has to be taken into account.

Imaging system here in SEM is based either on secondary electrons. Remember here, it is not transmitted electrons rather it is back scattered electrons. So, imaging system will be based on either secondary electrons which are generated by inelastic scattering events near the surface of the specimen or on primary electrons. These are the beams that are reflected or back scattered from the specimen. You would have two kinds of image

formation methods. One is on the basis of primary back scattered electrons and other on generation of secondary electrons or inelastic scattering.

Now, we will discuss here one of the detectors based on secondary electron detectors. Here is the particular detector electron beam. It is focussed by electromagnetic lenses through a small spot and is restored over the specimen surface. Secondary electrons generated during the interaction of the beam and the specimen is collected by detector. The amplitude which is the amplified signal from detector is displaced, displayed as brightness on cathode ray tube. It is which is being stood upon parallel with the beam. The magnification of the, will be determined by the ratio of diameter on cathode ray tube to the ratio of diameter on the specimen.

The secondary electron detectors which are in common use one is based on ever hard thoroughly design. Now, in these detectors a positively charged collector which is around 220 volt attracts the low energy which is less than 50 electron volts. The secondary electrons are then accelerated towards the scintillator by a potential of 10 to 12 kilovolt. The photons generated by the electrons striking the scintillator then enter a photomultiplier through a light guide. Here they are converted into photoelectrons and their number enhances almost by 10 to the power 5 to 10 to the power six times.

The resulting signal is displayed as brightness on cathode ray tube. The back scattered electrons have higher energies and directed mainly upward in direction of final lens. They are collected; they will not be collected by the secondary electron detector. If you have to use back scattered electrons then analog back scattered electron detector of a solid state type, or a scintillation photomultiplier type are placed just below the final lens so that way you can use.

The image observation is done by signal from the secondary or back scattered electrons and which is displayed as an image on the cathode ray tube or a video monitor. This is held in parallel with the electron beam scanning. The specimen for photography, the beam is scanned very slowly over the specimen in order to generate a strong signal. The image is recorded on a second high resolution CRT or cathode ray tube. Digital imaging is now a standard feature and high resolution video printers of laser video printers. It can be used and it could be kept on a magnetic or optical media or can be recorded on a

magnetic or optical media for computer processing and analysis. This was about the image observation.

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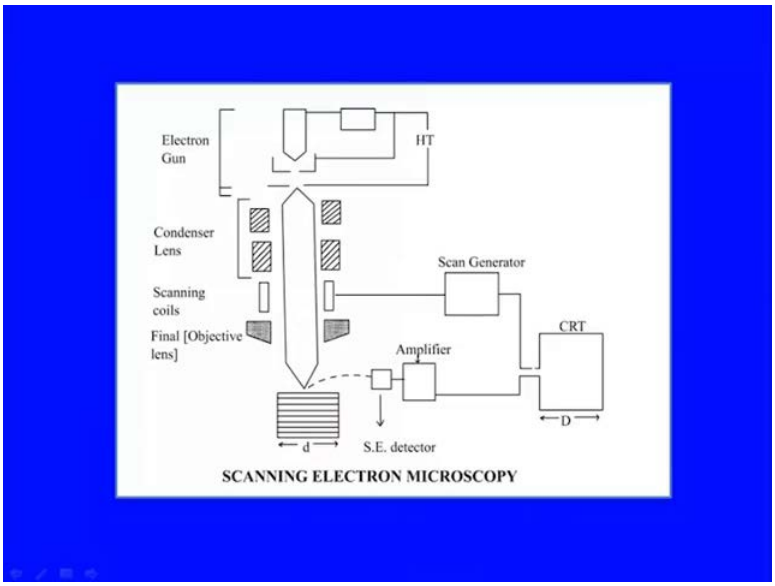

Now, let us see the optics. We have discussed this; let us try to understand through this figure. Like I said earlier, we have shown you electron gun but only difference is that it is 2 to 30 kilovolt anode. It is at a negative potential than anode. There is a difference here from transmission electron microscope. If you can see here rather than having a specimen at somewhere between the specimen is placed at the end of the column.

There are two condenser lenses here there. It is a scanning coil which scans the beam in parallel on cathode ray tube and there is a final lens. We call it objective lens. This specimen area could be tilted. This could be tilted at a certain angle towards the detector. Secondary detector is placed somewhere here. This detector which is secondary electron detector collects the secondary electrons which are of very low energy.

When it collects low energy electrons they need to be amplified to see the signal. So it goes through amplifier and through a photomultiplier tube finally, to the cathode ray tube. The ratio of this d to d on the specimen place is gives you the magnification. Let us see what is, how the detector looks like here. If you see in this figure this is the specimen which is tilted like I said. The specimen stage could be tilted here many fold in terms of 360 degree rotation, 90 degree tilting or move in x y direction or in z direction. There are, this is a versatile stage for accommodating various kinds of specimens big and small.

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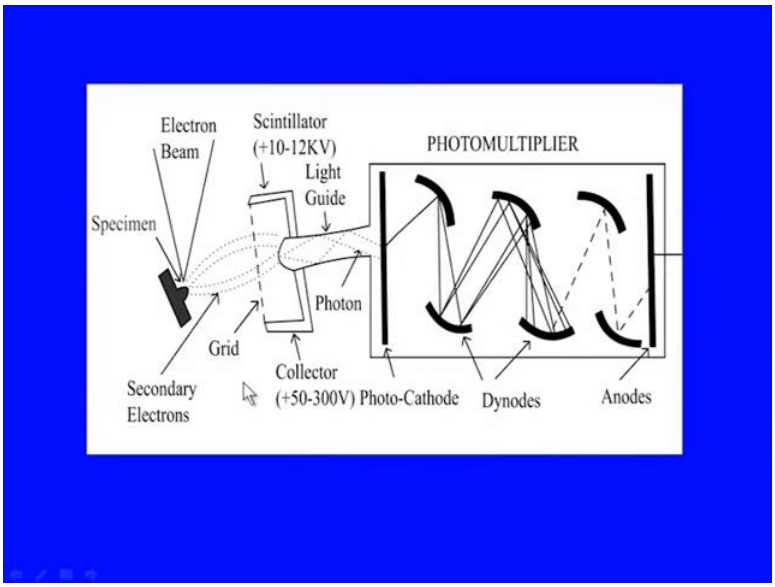

A specimen is here, little tilted electron beam is coming from the top. It is scanning, now this is a coated surface. It becomes conductive surface. The secondary electrons generated here, will be focussed or collected by a collector. This is a positive potential of around 50 to 300 volts. This collector attracts the electron and then they are passed into the scintillator. This is at positive potential of 10 to 12 kilovolt. These scintillators convert them into photons.

These photons will be then entering a photomultiplier through a light guide. This is a light guide here. Now, the photomultiplier, in a photomultiplier photons will be converted to the photoelectrons. Then through different dynodes and anodes, there is a photo photo-cathode here. The anodes will be directed towards the anode and these photoelectrons through these system will be amplified many folds. The signal can be clearly seen on the cathode ray tube. This is how the secondary electron detector works. This was about the optics of scanning electron microscope. One of the detectors we have discussed is secondary electron detector. The main part of this whole microscopy technique is that.

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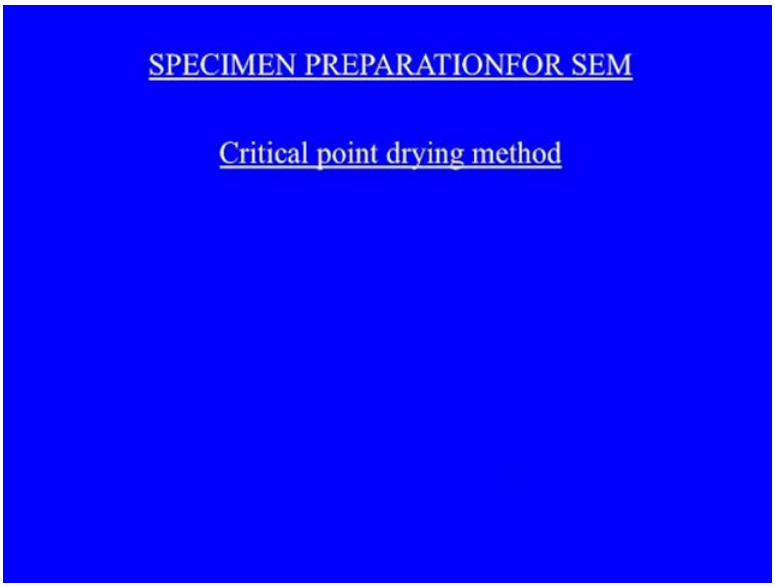

What we are viewing is the surface details. How to preserve those surface details while processing or making the specimen for the microscopy observation. In SEM a 3D view of biological specimen which could be cells in culture or any other biological specimen for that matter will be imaged. Special preparative procedures are needed to preserve surface details of the specimen and make them visible. Let us see how this is done.

Now the specimen must be dehydrated before placing them in the instrument. We have told you earlier because of the high vacuum conditions. If you dehydrate the specimen the surface details will be lost. This is because of the surface tension problems. What happens is, that normal procedures where air drying or alcohol dehydration is followed by evaporation of solvent.

It is employed, it will produce surface tension forces that will distort the cell shapes and disrupt delicate membrane structures. Now, defects on the surface tension can be avoided. You have to make sure that surface details are not lost on what you are trying to view. How to do that? We have to avoid the effects of surface tension. This could be done by using one of the methods called critical point drying method.

Now, what is this critical point drying method? When a solvent is in equilibrium with its vapour phase it is heated to a critical temperature and subjected to its critical pressure. That is we call critical point. The densities of liquid and vapour phase are equal and therefore, the phase boundaries disappear. When the phase boundaries disappear the

transition from one phase that is from liquid phase to vapour phase is done without any problems of surface tension. This is how you do not develop a surface tension.

Now, if the temperature is held little above the critical temperature the vapour can be exhausted without condensation. The specimen is dried so what you are trying to do is you are trying to preserve the surface details by critical point drying. How to do that actually and which particular solvents are needed to be used? Now, here for critical point drying liquid carbon dioxide is the commonly used solvent, here for critical point drying why because its critical temperature is 31 degrees celsius. Its critical pressure is 10 a t p s. This is readily achievable without the risk of damage to the delicate biological samples.

Now, compare it with water, the critical temperature and pressure of water are 374 degrees celsius and around 3184 p s i. This cannot be used with biological samples. So, the liquid carbon dioxide is used where first the samples are exchanged. The water is exchanged for liquid carbon dioxide. Then at critical point they will be dried to avoid surface tension problems. To preserve the surface details after drying surface of the specimen will be made conductive in order to dissipate the charge. This builds up as electron beam is stood over the specimen. The specimen, first part of the specimen preparation was critical point drying. Once you have dried it and preserved all different details you have to quote it with the metal heavy metals.

Now, for most applications in which surface topographic information is sought and low to moderate magnification. It is required; the surface is quoted with a metal such as gold in a spatter coater for higher magnification. Gold palladium alloy and other metals like platinum chromium etcetera are used which have a smaller grain size. Gold has a grain size of from 50 to 40 nanometre. This is a little higher, which is a little more and could not be used for higher magnification.

Now tungsten and tantalum with fine grain size can be deposited in thin layers for much higher magnification. Here, you will use again a vacuum evaporator equipped with rotating and tilting stage. For doing this, let us see how it is done. If the specimen will be used for back scattered electrons imaging of gold labelled antibodies this could be utilised and it could be made conductive.

Now, let us see how this is spatter coater is utilised for coating the specimen. What is done is that specimen is mounted on aluminium step with conductive tape. It is placed in a spatter coater the chamber is evacuated. The argon gas is introduced to maintain a pressure of about 6 to 7 Pascals. The spatter coater has a ring shaped cathode or target made of metal to be spattered and a ring shaped anode between the cathode and specimen. Application of high voltage generates plasma of ionised argon and positively charged ions bombard the target knocking out metal atoms. They are scattered in all directions as they collide with arginines and electrons. The surfaces of the specimen are evenly quoted by multi directional metal atoms.

Now, heat damage to specimen could be avoided by a cooling the specimen using a magnet to deflect electrons. Apply high voltage in pulses rather than continuous rays. You can do, you can use any method to avoid heat damage. This is how the specimen for scanning electron microscopy is prepared. This completes our section on scanning electron microscopy.

Now, like we said both transmission and scanning electron microscopes are valuable source for lot of information which cannot be observed otherwise. They have helped in increasing the resolution manifold so that minute details of different specimens which could range from biological samples to other samples could be imaged. There are lot of applications of electron microscopy like light microscopy.

In light microscopy you have a resolution limit. That limit has been crossed in or overcome in the electron microscopy. Various applications are kind of the same. You can examine isolated and cultured cells in both transmission electron microscopy and scanning electron microscopy. This is done by preparing samples in which you can examine sub cellular fractions by negative staining or thin sectioning.

You can find the precise location and distribution of molecules or cell components to understand physiological function by imino labelling. This can be done by pre embedding or post embedding the imino gold labelling. Lot of different things can be visualized proteins, macro proteins, nucleic acids, proteins and nucleic acid interactions, various macromolecular assemblies. It is like you have different applications of electron microscope and it is being used in different areas of biotechnology. Even in non biological fields, now I will show you some of the figures.

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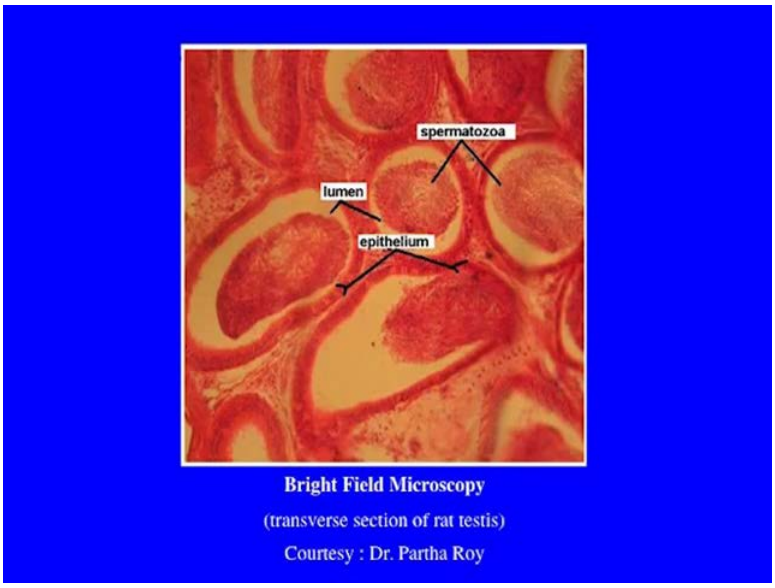

This has been kindly provided by my good friend and colleague Doctor Partha Roy. I thank you, for these figures. Let us see these figures. Here, are some images and you can see lot of these images in books and the internet. This is one figure which has been taken by bright field microscopy. You can see here, clearly the spermatozoa, lumen and epithelium. Different sections have been labelled in here. It is a transverse section of the rat testis. So, this is a bright field microscopy.

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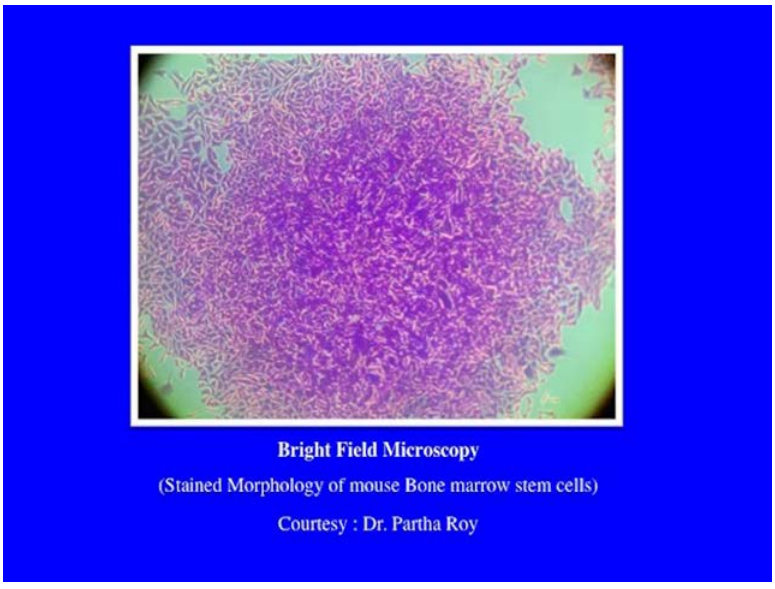

Now, let us see some of these other images. This is again a bright field microscopy of mouse bone marrow stem cells. It is stain morphology. You can see here, the a particular pattern of mouse cells

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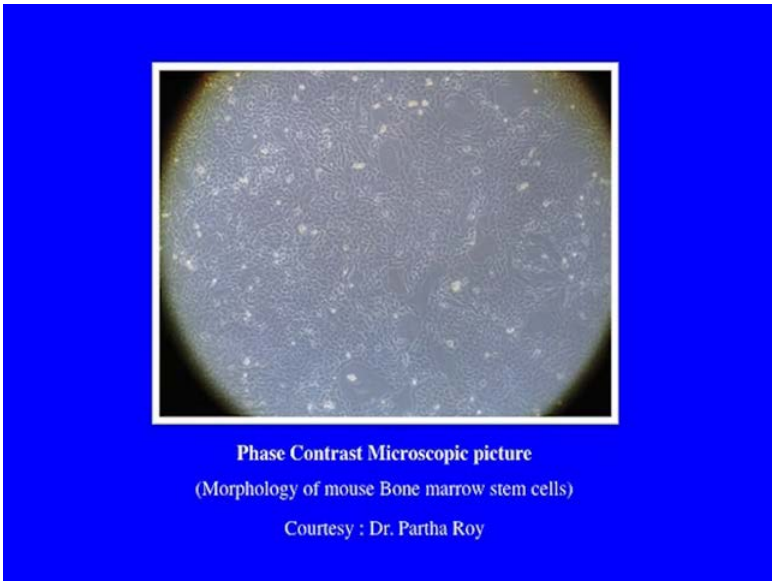

The same image has been taken in phase contrast. You can see little without staining. You can get lot of details in phase contrast picture. Also, here this is same bone marrow stem cells of mouse.

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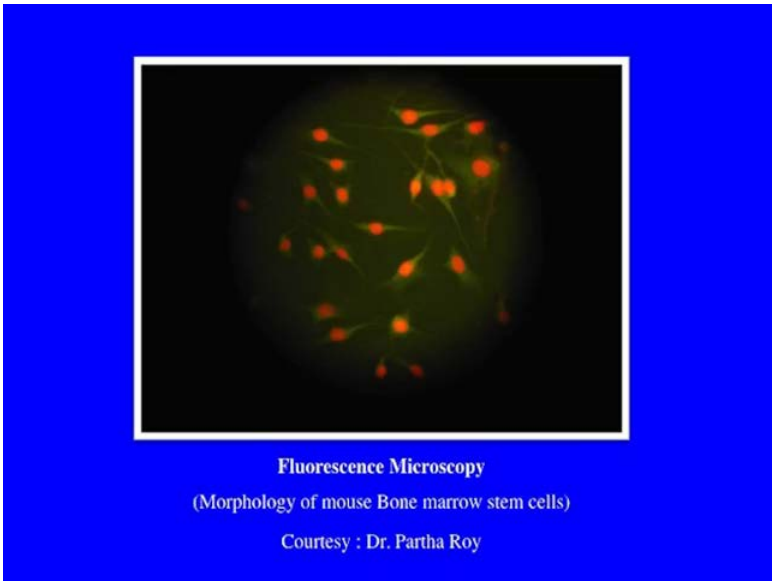

The same bone marrow stem cells have been done in fluorescence micrography. You can see these cells in here which are fluorescing well. They have been stained with fluorescence dyes here.

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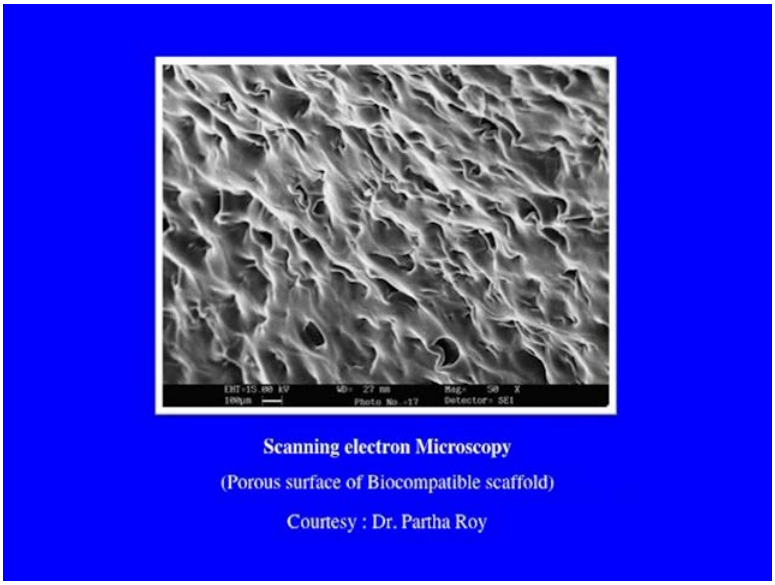

This is a scanning electron microscope picture. Here, is of a porous surface of biocompatible scaffold. All pictures have been provided by Doctor Partha Roy. They have been taken in his lab. Thanks to him. Here, we complete our section on the microscopy. I hope you have understood the basic concepts and details of different microscopy techniques. We have discussed in here, we have gone through light microscopy techniques.

We have discussed many various basic concepts of microscopy technique. Then we have gone through techniques like bright field dark field face contrast differential interference, contrast fluorescence, polarization microscopy in light microscopy. We have discussed electron microscopy techniques which are transmission electron microscopy and scanning electron microscopy.

Now, I think all of you must have been benefitted by this particular course. I would suggest you should read more and this will certainly inculcate curiosity in you. I would say that both microscopy technique both light and microscopy was started with light microscopy. It has allowed us to view the tiny world of cells microorganisms. This tiny world has become much bigger, maybe much bigger than our world. It has been made

possible and all the information and knowledge which we gained from this could be utilised for many different application in the field of biotechnology.

You can apply it for in agricultural sciences. You can apply it medical sciences, forensic sciences and non biological sciences also. Here, the microscopy technique has allowed us and has empowered us to understand various phenomenon's which are going at some microscopic label. To manipulate them and to understand them this has been a great technique to work with.

So, this completes our section on microscopy. Now, we will move on to the next important technique in biotechnology. In this course that is radio isotope technique it is a highly sensitive technique and it utilised in various branches of the biotechnology. I hope you understand all the different concepts. As we have gone through some lectures and I hope you will keep listening to these lectures throughout.

Thank you very much.