Analytical Technologies in Biotechnology
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Module - 1
Microscopy
Lecture - 6
Transmission Electron Microscopy

In the previous lectures, we have discussed about the basic concepts and certain detailed techniques in the field of light microscopy. Now, as we have gone along, we have discussed all the different concepts and certain techniques to create contrast for visualization and light microscopy. If you could recall we have gone through a lot of different techniques, where you require staining or you might not require staining for visualization.

Now, remember light microscopy has allowed us to view into the tiny world of cells microorganisms, macro-molecular assemblies and so on. It has allowed to ((Refer Time: 01:14)) world- tiny world, it was totally unknown and unseen has been allowed to be viewed by us with the advent of the light microscopy. But, light microscopy has certain limitations. Now limitation is due to the wavelength of the light which cannot be reduced. So because of the wavelength of the light, the limit of the resolution is also limited, that is we have discussed earlier that you can only see or you can go up to say little better than 0.2 micrometer or 200 nanometre. Details which require further higher resolution cannot be seen in here. This particular bottleneck could be for somewhat, could be solved by utilising little lower or you can say wavelength with in ultraviolet region.

But, this particular problem had been sorted out in electron microscopy. In the coming lectures, two lectures we are going to discuss about electron microscopy technique and light microscopy. The basic concepts and lot of things might be similar, but there are specific differences between light microscopy and electron microscopy. We will be dealing with those things as we go along in the electron microscopy section.

So, let us start with this new technique which we are going to discuss today. In the next lecture, that is electron microscopy. Now, microscopy technique, this particular technique which is electron microscopy technique is one, where a beam of electrons is

used for illuminating a specimen to generate a magnified image. In the light microscopy we used light to eliminate the specimen.

The basic principles of electron microscopy are similar to light microscopy. But, there are differences in terms of like say; one major difference could be the lenses in the light microscopy, if you could recall they were glass lenses or optical lenses were used in the light microscopy, but in case of electron microscopy electromagnetic lenses will be utilised. These are used in focussing a high velocity electron beam in place of visible light. When we say electrons needs to be used here then electrons cannot be the beam cannot be operated in the open condition.

The entire tube containing the different components of electron microscopy has to be maintained under an ultra high vacuum. This entire tube which contains electron source the lenses, the viewing screen everything is enclosed chamber. Therefore, because electrons are absorbed by atoms of air so this will be placed in an ultra high vacuum. The electron microscopes provide much greater resolution. I was stating that limit of resolution is a problem in light microscopy. You cannot go below a certain limit of resolution. But, here in electron microscopy there will be much higher resolution. This could be achieved in light microscopy. The wavelengths of electrons are almost 100,000 times shorter than visible light, which are the photons.

This effective resolution limit of electron microscope is considerably less than that of the particular theoretical achievable resolution that is 0.005 nanometre. This is because of the problem of spherical aberrations. As a result, the electron focussing lenses suffer. To solve this problem the n a of the lens is made very small from 0.01 to 0.001. You can see that, theoretically light microscope; the electron microscope can give you much higher resolutions. But, because of certain spherical and other problems their resolution is lower. Still it is much higher than what you can obtain or achieve from the light microscopy.

Now, advance electron microscope can achieve resolution which is better than 50 pico metre or 0.5 angstrom. This is at magnification of around, we can say one million x or more than that. So illusion limit for light microscope if you could recall is 200 nanometre and it is useful for magnification is below 2000 x. If you could see here, that in light

microscopy you cannot go beyond a certain limit magnification. Here, you can magnify the image even after obtaining from the objective lens manifold.

This is because the amount of details, which is available in a electron microscopy image after objective lens is much more. You benefit from magnification rather than blurring of the image or distortion of the image. What are all the advantages which we were talking about the electron microscope? One of the disadvantages of electron microscope is that, only non living samples can be observed.

The high vacuum conditions are required, like I told you, to have better than 10 to the power of minus 4 torr of the vacuum conditions. When you have to maintain the vacuum conditions, living samples cannot be utilized. As we go along, we will be discussing about the differences. Also, I will show you how we can compare a light microscope and an electron microscope. Now, in this section we are going to discuss about the two main types of electron microscopy techniques.

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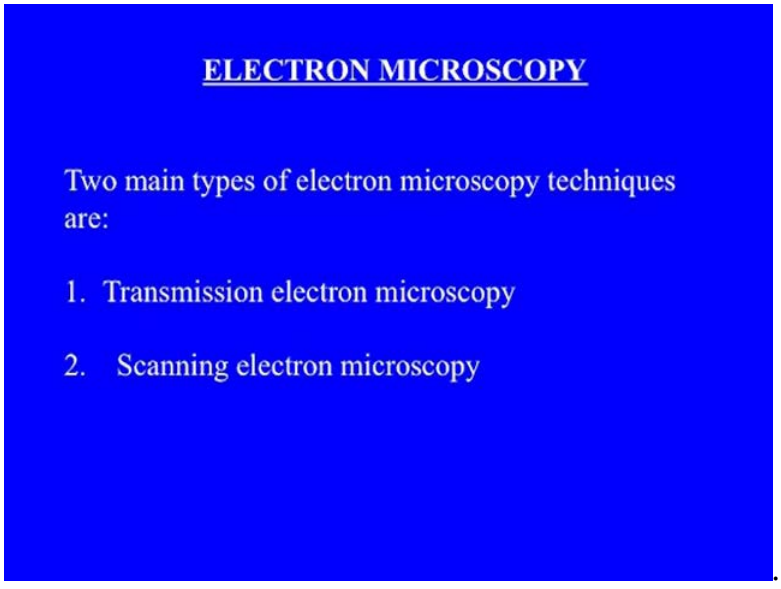

These are transmission electron microscopy and scanning electron microscopy. Transmission electron microscopy, the images are formed by the transmitted electrons. It provides you the internal details of the specimen in contrast scanning electron microscopy. Here, rather than transmitted electrons, back scattered electrons are used for image formation. Rather than internal details you look for, or what you see is the surface details which are provided by a scanning electron microscopy technique. Let us move on.

What are different components of electron microscope? I will introduce these things. Then we will go in detail and discuss each of these components.

Electron microscopes like I said, it has to be an enclosed chamber. It consists of a hollow cylindrical column within which the electron beaming is confined. There is a console having dials which are electronically controlling. The operation of the microscope is from the top to down. Here you have to remember that the column contains the cathode emission source. This is the emission source, which is at the top which is heated and acts as a source of electron. If you could see, if you could recall illumination source in the case of light microscopy, below that is at the base of the microscope. It is at the top of the microscope.

You can say the electron microscopes in comparison to light microscopes are upside down. Now electrons are accelerated as a fine beam by the high voltage applied between cathode and anode in high vacuum conditions in the column. They are brought to focus by powerful electromagnetic lenses. These are different from optical lenses. The size of the beam is controlled by various apertures, where the condenser lenses focus the beam on to the specimen magnified image is formed.

This is done by the objective which is further magnified many times by the projector lenses. We call it the light microscopy ocular lenses. Finally, the image is focussed onto the viewing screen that is fluorescent screen. You can then see the screen directly or indirectly. These are the various components of the electron microscope. Now, how do you distinguish them? Let us see. Then we will be going in detail.

First is the illumination source or system. The illumination system is made of one electron gun. What does the electron gun do? It generates electrons and provides a coherent electron beam. Condenser lenses, there are two condenser lenses one and two which determines the smallest illumination spot size on a specimen. The amount of illumination in terms of intensity, there is the condenser aperture which reduces the spherical aberration. It also helps to control the amount of illumination striking the specimen.

The illuminating system is made up of electron gun condenser lenses and condenser aperture. There is a specimen manipulation system or you can say the specimen stage. There are specimen exchanges and specimen stage which is provided for moving a

specimen inside the column of a microscope. Remember, the microscope is evacuated in high vacuum conditions. Hence there has to be method to introduce your sample through an air lock system into the column of the microscope.

There is an imaging system which is made up of intermediate apertures objective lens and projector lenses. They will focus the image onto the screen and finally, the observation and camera system. There could also be a viewing chamber, which contains the viewing system of a final image. There could be a camera which could be utilised for recording the image, so these are various components.

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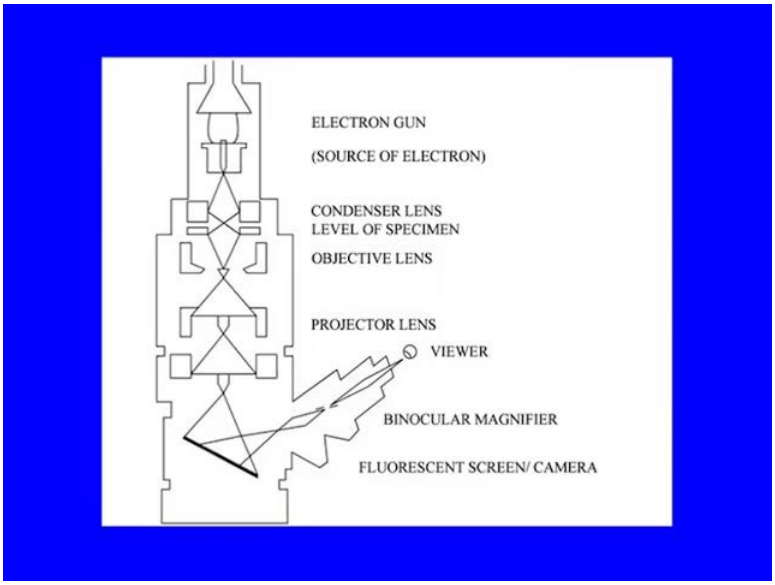

Now, if you see this figure here, this figure shows certain various components. You see that the source of electron is on top of the column. This will be formed of cathode anode and the apertures. These are condenser lenses. Here, a label of a specimen is present in here. After the specimen, there will be objective lens which is not written here. After the objective lens there will be projector lenses which will finally, emit or form the image onto the fluorescent screen. This fluorescent screen you can see, it will directly or you can see it through binocular magnifiers. This is the electron microscope with various different components.

Let us see how an electron microscope compares with light microscope. This is just to give you a feeling of the light microscope and electron microscope. Here, we have just to align them. This is also upside down light microscope. If you see here, the light

microscope lamp is a halogen lamp or certain for the simple light. This is a filament which is for producing electrons. The cathode filament is used for producing electrons. Now, the lenses if you see, these are glass or optical lenses. Here, these are electromagnetic lenses. Likewise, you have similar patterns.

Here, the condenser lens is there which focuses or is a part of illumination system. The condenser lens in electron microscope will focus the beam then you have specimen place. The specimen will be placed in the objective lens. It will take information from the specimen like in light microscopy. These are the objective lens, again electromagnetic lenses.

Then you have projector lenses which are more than one in case of light in the electron microscope. Finally, we have the image formation. We can say the basic arrangement is same, but the kinds of lenses which are used kind in illumination are different from light microscopy. Now, let us move on to the transmission. Like we said, we are going to discuss two techniques here. One is transmission electron microscopy and the other is scanning electron microscopy.

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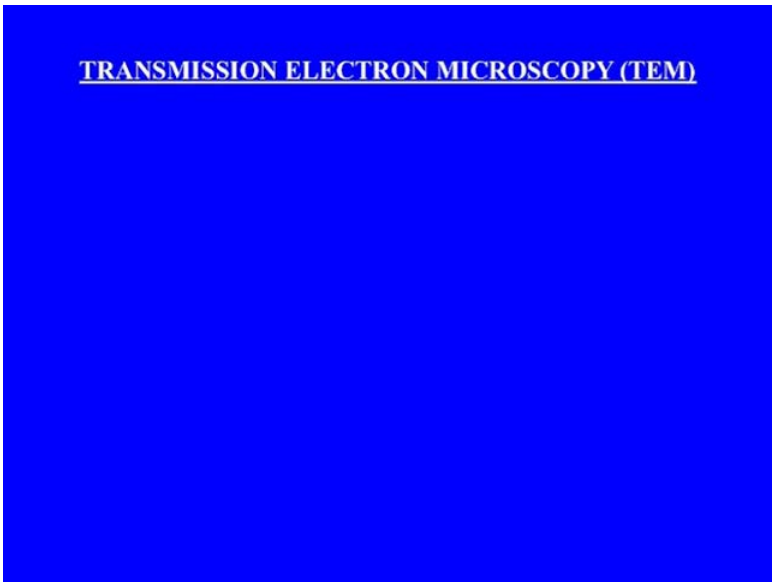

Let us start with transmission electron microscopy. This particular technique or microscopy technique is one where an image is generated from the interaction of electron beam. It is transmitted through an ultra thin specimen. The magnified image is focussed onto an imaging device which could be photographic film, a fluorescent screen or even a

CCTV camera. TEM provides much greater resolution than the light microscopy. As we were discussing the wavelength of electron is almost 100,000 times shorter than the visible light.

The resolving power of this microscope is the TEM stems from the wave properties of electron. What happens is that, the wavelength of an electron is not a constant distance like we had in the light microscopy. It is rather, it is related to the speed at which the particle is travelling. This in turn depends on the accelerating voltage applied on microscopy. This is the potential difference or the voltage difference between the cathode and anode. The speed of the electron will depend on that.

The wave properties will depend on the accelerating voltage so it is determined or the wavelength will be determined by square root of 155 volts or v. You can calculate as you know the accelerating voltage. The standard transmission electron microscopes operate in the range which lies from 10,000 volt to the 100,000 volts at around 60,000 volt the wavelength of an electron. It is approximately 0.05 angstrom.

So, providing theoretically we can say an excellent limit of resolution which could be almost 40,000 times better than the resolution of light microscopy. The problem like I said, there are problems of spherical aberrations and others. The effective resolution limit of electron microscope is considerably less because of these problems. The electron focussing lens will suffer and to avoid this numerical aperture is made very small. The effective resolution which could be achieved by the electron microscopes is somewhere around 0.5 Angstrom. Angstrom magnification like I said, more than million times magnification can be achieved without any problem.

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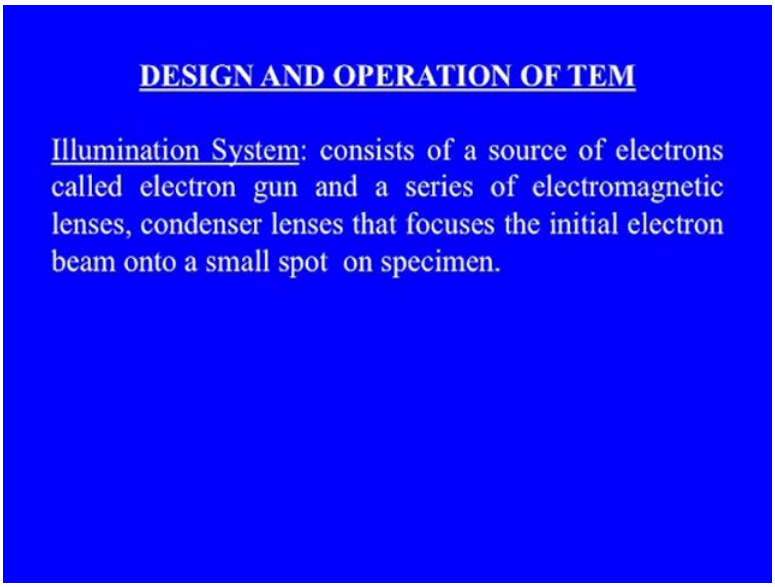

Now, let us get into the design and operation of transmission electron microscopes. We were discussing the different components. Let us discuss them in detail. The illumination system like I said consists of the source of electron called an electron gun. A series of electromagnetic lenses, these are condenser lenses that focuses the initial electron beam onto the small spot on the specimen.

Now, the electromagnetic lenses like we were discussing these are constructed of soft iron core and these soft iron cores would with an electrical conductor. This produces a magnetic field which is utilised to focus a beam of electron. Let us see what is an electron gun is. Now, different components of electron gun include cathode. A cathode is also called emission source or filament. This is maintained at negative potential difference to anode almost like 50 to 100 kilovolt negative potential difference.

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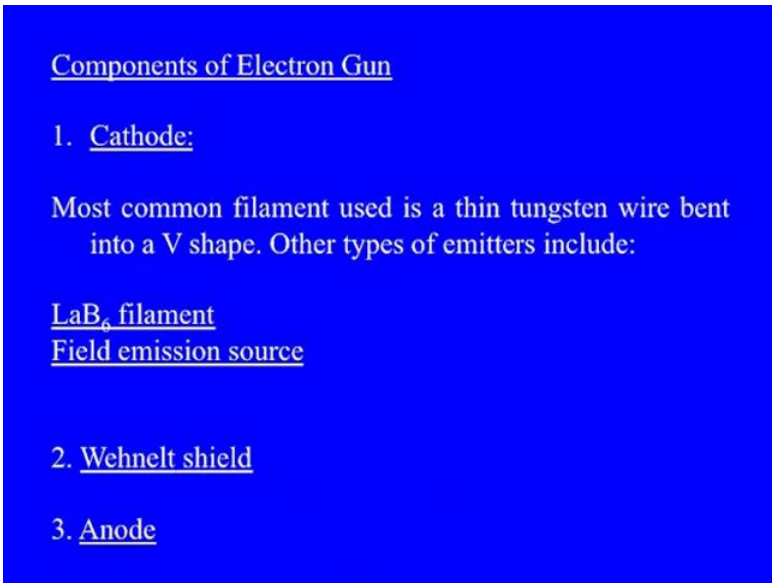

The most common filaments used are thin tungsten wire. This is bent into a v shape, that is the most commonly used and cheap one as well. But, there are other types which can give you better illumination or a fine focus. These are lanthanum hexaboride L a B 6. The field emission source, now lanthanum hexaboride filament is a pointed rod that provides a stable current from a small area than a simple tungsten filament. It is almost ten times brighter than the tungsten filament. It has a lifetime of 500 hours or longer. But, it requires a vacuum in order of 10 to the power of minus 6 torr.

You would have, to have better vaccum, but this is much better emission source as compared to simple tungsten filament. There is another better source than your lanthanum hexaboride and simple tungsten filament that is called field emission source. You must have heard about field emission electron microscopes. This requires very high vacuum conditions like 10 to the power minus 9. It operates at room temperature. It requires a single crystal of tungsten within emitting region of approximately 10 nano metre brightness.

It is almost 1000 times that of standard tungsten filament. It employs double anode first anode around 2000 volts. It extracts electrons from tip and the second anode accelerates the electron down the column. It produces a very small probe size with negligible energy spreads. It is very effective and very good source of electrons. You can say this is the

best, one of best field you can say emitter or filament. Here, emission source it is certainly more expensive as compared to a simple tungsten filament.

There is another component which needs a mention here. That is called the Wehnelt shield. The Wehnelt shield is positioned between cathode and anode. It is held at potential difference of 100 to 200 volt negative to cathode. Remember it is negative to cathode. What it does, is the function to create intense cloud of electrons in a very small aperture. You can say it serves as an electron source. So electrons from cathode like kind collects in an intense cloud of electrons at this particular aperture.

Wehnelt shield is connected to high voltage and to filament heating voltage by resistors. Then there is anode which directs the beam of electrons. Now, transmission electron microscope will employ two condenser lenses with an aperture. There will be an aperture of around 100 to 200 hundred micrometer. This is placed between the second lens and the specimen to reduce the spherical aberration. There will be deflection coils which are used to shift and tilt beam and these are part of the illumination system.

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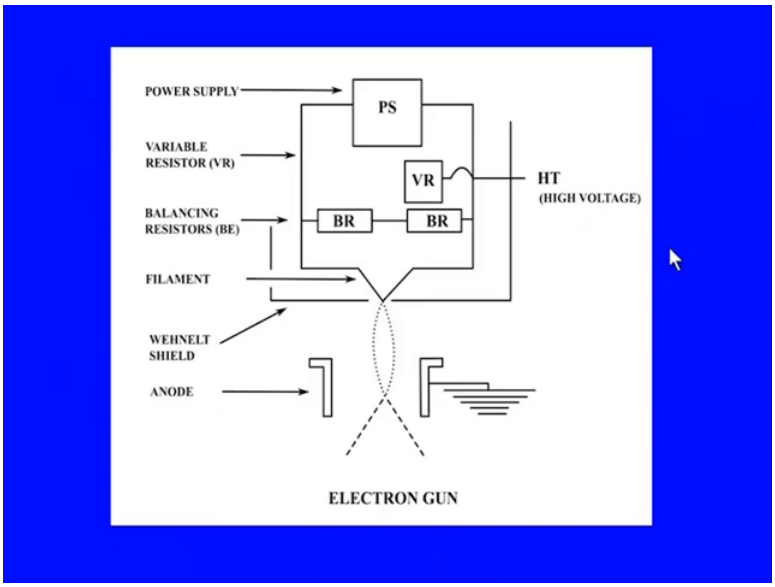

Now, let us see how this looks likes. This is an electron gun or emission source. Here if we see this figure there are lot of different electrical things power supply variable resistors balancing resistors. What we are interested in are the, cathode and anode. The cathode is this simple V shaped tungsten filament. This is when heating gives you electrons.

The anode is here, which because of there is a potential difference. The electrons will be rushed or directed towards the anode from cathode. Wehnelt shield condenses. These result in making intense cloud. Here, this seems to be the electron source. Then finally, the electrons are moved down the column through different lens system through the specimen to form the image. This is like a typical schematic of electron gun which is employed in the electron microscopes.

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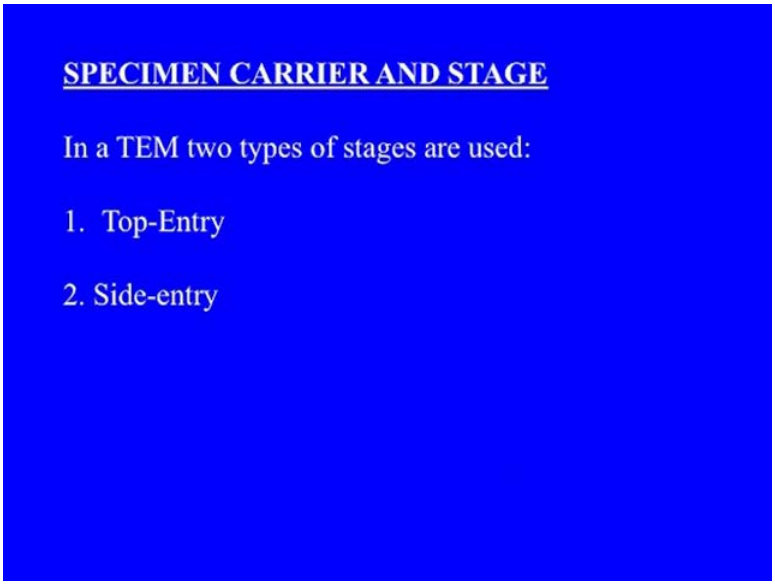

Now, the second part which is after illumination system is the specimen carrier or specimen stage. This is where the specimen is placed. Now, transmission electron microscopes have two types of stages. One is called top entry which utilises a cartridge like specimen holder. This is inserted into a stage from above. If you might have seen a simple printer, actually where cartridge is inserted from above it is something like that where it is a top-entry stage. There is a side-entry stage which utilizes a rod shaped specimen holder with one or two places for specimen. At one end it is by kim and spring mechanism the rod is inserted into stage through an airlock on one side of the column. Like I said, it is an evacuated chamber.

So it has to be inserted, that vacuum is not disturbed now although top-entry stage provides slightly better resolution. This is because it fits better. But, in TEM side-entry stage is mostly utilized. It allows more versatility in positioning of the specimen and design of device for tilting specimen. It is also very simple. It is much more convenient

and more used in here. In TEM or transmission electron microscopy the stage motion is limited to plus minus one millimetre in x y direction. If there is a goniometer fitted in the stage then specimen could be tilted up to plus minus 60 degrees. These are limited motions which can occur in the specimen carrier or specimen stage to focus to place your specimen.

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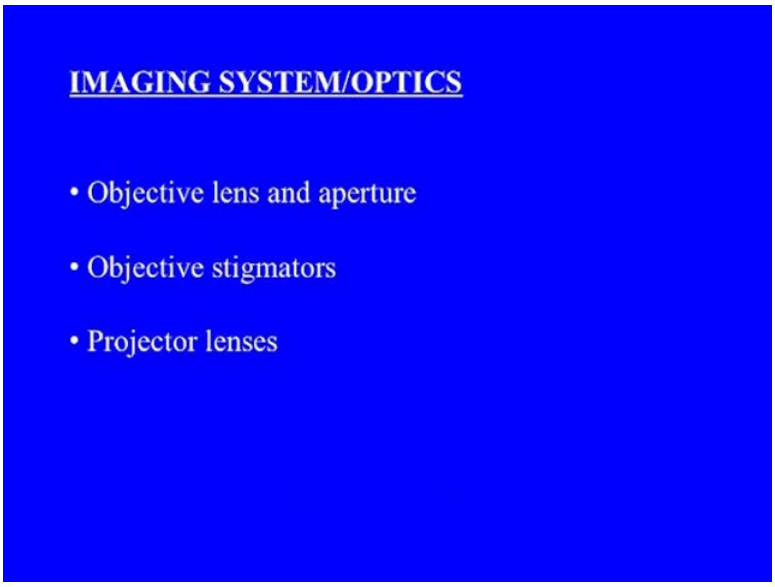

After specimen stage comes the imaging system or optics. This will form the final image. This consists of objective lens, aperture, objective stigmators and projector lenses. Like in light microscopy the objective lens will collect or the beams will pass through the specimen. It will be focussed by objective lens. Objective lens will magnify the image up to 100 x. The focal length of objective is very low around 2 millimetres. The specimen must be placed within the concentrated magnetic field of the lens. Varying currents to objective lens permits focussing of image. Then the projector lenses which could be 2 or 3, they are located below the objective lens.

Now, the final magnification as we have discussed it could be from 1000 to maybe million times of the image. It could be varied by again altering currents in projector lenses. Like I said, there are sufficient details present in the image from the objective to magnify it. It manifolds to get information unlike light microscopy. There are objective apertures below specimen which eliminates the electrons that have been elastically scattered.

Generally apertures of around 30 to 50 micrometer in diameter are utilized. There are stigmators like I said, objective stigmators are special type appliances. They are used to compensate for certain imperfections which are present in the construction and alignment of the column. We are not going in detail of those lenses here. So these are part of the imaging systems or we call it optics.

Image observation and recording, now images can be viewed on screen with projector lens. It will focus the magnified image onto the fluorescent screen which could be light emitting phosphor or fluorescent surface screen. It can be observed directly or for increased magnification you can critical focus. You can observe it through binocular microscope. Image is recorded on sheet film camera mechanism. It located beneath the screen a video camera. It can be used or a charge coupled device could be used to record digital images that can be used to be stored in magnetic or optical storage media.

Now, computers can be employed for viewing images, processing and analysis of it. That makes it much more convenient to process these images and analyze them. This was about image observation and recording. Now, after like we have discussed about the various components. One major aspect of the electron microscope is microscopy technique. It is the specimen preparation. Why it is so important? The scattering of electrons by specimen component of specimen is proportional to the matter present. We call it mass thickness or atomic density.

We call it the measure of number of atoms per unit area and their atomic density. What does that mean? To understand this, if we see biological materials which are mainly made up of atoms of relatively low atomic number like carbon oxygen, nitrogen, hydrogen etc. These are therefore, not effective scatterers of electrons. This is because they have relatively less atomic density. Mass thickness or atomic density of a specimen has needs to be enhanced. To enhance them we need to fix and stain the samples with solutions of heavy metals.

Then you can get much heavier metals to diffract or scatter the electrons. To obtain the contrast unless you can obtain the contrast you know that you cannot see the images. They bind these heavy metals; they bind differentially to different components of the specimen. The parts which complex with the metal atoms will not be allow the passage

of electrons, wherever there is a concentration of heavy metals. What will happen to the electrons that will not pass through?

The other parts where the electrons or heavy metals are not there they will allow the passage of the electrons. That is, the transmission of electrons will be allowed to the screen. What will happen? The screen is hit by the electrons due to the passage through the specimen. The areas will look bright, but wherever the electrons have not been allowed to pass through. The areas on the fluorescent screen will be dark. That is how the contrast will be created and the image formation in the electron microscope will take place. This requires that you have to prepare specimen in order to provide the scattering capacity.

Here, since it has to go through so much of processing, like I said. It is a vacuum system ultra high vacuum system is present in the electron microscope. Only non living specimens can be observed in here. Except for certain environmental microscopes where they allow partially hydrated samples where the vacuum conditions are around 10 to the power minus 2 torr.

In high vacuum conditions, the hydrated samples, the living samples are not allowed. That thickness of the specimen for transmission electron microscopy must be less than 100 or 200 nanometre. This is because of poor penetrating power of electrons. This is much thinner than light microscopy sections. Various methods of specimen preparation in TEM for introducing contrast are discussed in this, as we go along. We will discuss about various ways to create contrast.

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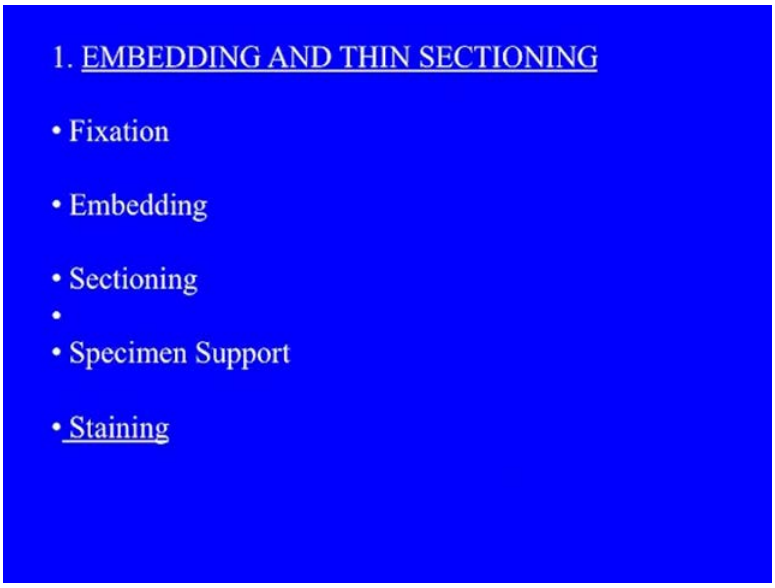

Let us see the first method. If you could recall the specimen preparation in light microscopy involved fixation dehydration, embedding and sectioning. Here also, we follow the same steps with certain differences. The first is the fixation. Now, for optimum reservation of organals and membrane structures a strong protein cross linker is used. A fixative can also be used such as butaldehyde. Now, butaldehyde is a strong cross linker. If you would like to retain some amount of biological activity like antigenicity or so a weak cross linker such as paraformaldehyde may be used.

Samples which are used for morphological studies may routinely be post fixed. That is, you have fixed it with certain fixatives, but you can postfix with osmium tetroxide OsO 3. What it does is, one thing it is heavy metal and another is it will cross link certain things like this highly reactive metal oxide. It will be predominant and dominantly react with unsaturated lipids. It will provide electron density. It will impart the atomic density or electron density to biological membranes. It will also reacts with proteins, lypo proteins, nucleic acids and other things.

This will, this can be used after fixation. Then potassium ferri or ferro cyanide can also be added to increase the membrane contrast. There are lot of other things which could be used. After fixation many times, the samples may be treated with solutions of heavy metals. This is not a staining state. It is like you, just after staining what you are doing is like, you are trying to provide more and more atomic density for scattering. This is not

compulsory. Now, uranyl acetate and lead aspartate plus bismuth, some nitrate can be used as blocked staining reagents here.

So, after fixation the embedding is done. The first thing you have to do in the embedding is first you have to dehydrate the samples. As many of these raisins which are going to be utilized are water emmissible, so the most widely used are epoxy raisins in addition to epoxides like epon or epon substitutes. These are used there, and like other substitutes are all there for enhancing the reaction or for hardness. They are utilized like for example, n hydrides like nadic methyl, n hydride called n m a.

They do diphenyl succinic n hydride d d s e are added to raisin to modify its hardness. An accelerator like d m p 30 could be utilized to speed up the polymerization. These are other additives which can be used for enhancing the polymerization and to control its hardness. Like I said, this has to be done. The dehydration has to be done before we start that. This could be done in a similar way. It has been done by a series of ethanol water.

Finally, absolute ethanol and here, propylene oxide is generally used as a transition solvent. There could be other raisins which are their names; the commercial names are l r white or l r gold. It could be used and these have low viscosity. They are acrylic raisins and could be used for post embedding for imino cyto chemistry. These are other applications and these could be used for embedding.

Once it is embedded and hardened, then sectioning is done. In sectioning if you see, if you could recall a microtome was utilized in light microscopy. Here, you need much thinner sections. Here, an ultra microtome is used to prepare sections from 50 nanometre or less to 1 to 2 micrometer in thickness. These sections are cut by either glass knives or diamond knives in particular. These sections float of the edge of the knife onto the water trough. The water is held in a trough behind the edge. These floating sections are collected onto specimen support. I will show you that here, for staining and examination in TEM.

Now, specimen support is a grid which is called specimen grid. It is around 3.05 millimetres in diameter. It is made of fine metal mesh usually copper. It can be other metals with openings in metal bearing from 450 to 20 micrometer in case of acrylic raisins. The grid is first covered with thin plastic films and sections are collected on a supporting film. I will summarize this whole thing a little later.

Then staining, if you could recall we have done post fixation. There was osmium tetraoxide and then also block staining reagents were used. Still after the embedding and cutting thin sections again staining is done to impart more scattering power. Now, the sections require additional staining with solutions of heavy metal to further increase the contrast. The two most widely used stains are uranyl acetate. It is used in acetic conditions and there is a lead citrate which is in basic conditions.

This will give uranyl acetate gives, mostly gives contrast to nucleic acid and lead citrate. It could be utilized for contrasts of lycogens and ribo nuclear proteins etcetera. The staining times could range anywhere from 1 to 10 minutes. Then different sections could be thoroughly rinsed in distilled water and then can be dried and can be used for observation under electron microscopy.

So, this was the extensive specimen preparation in the electron microscopy which is transmission electron microscopy. There are other methods of creating contrasts. As we go along, we will discuss that. But first we should summarize whatever we have the specimen preparation and so I would like you to focus on your screen. Here, alright so till now we have discussed about transmission electron microscopy and its various components. Let us summarize how specimen preparation is done. First thing is you will defect or get the tissues or whatever sample you have and then that sample has to be.

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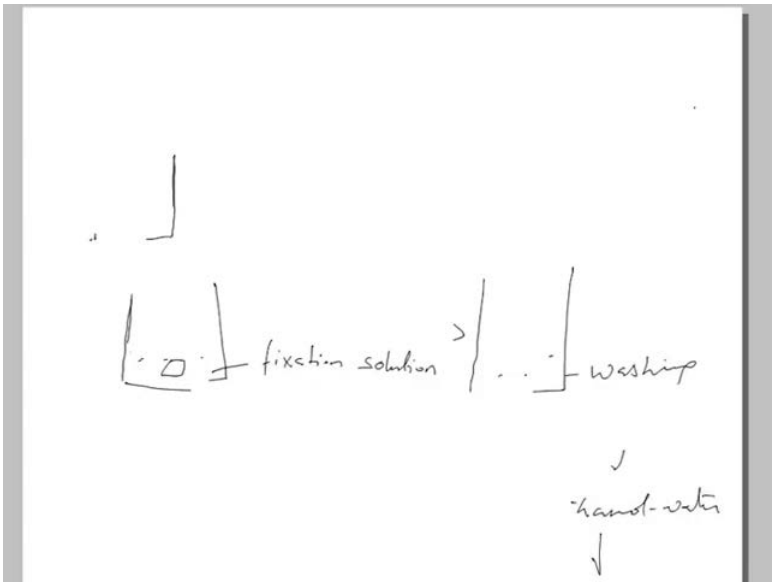

If I say that particular sample needs to be the fixation. It has to be done now. The fixation in that sample will be done. So, you have the material in here. This is the fixation solution. Fixation solution like I said could be anything. It could be simple paraformaldehyde or butaldehyde. Now, once you have done this the fixation solution, you will remove this. You will, you have to wash it. You will do the washing and you will remove any after fixation. This step has to be carried on. After that the most important part is you have to go along dehydration step.

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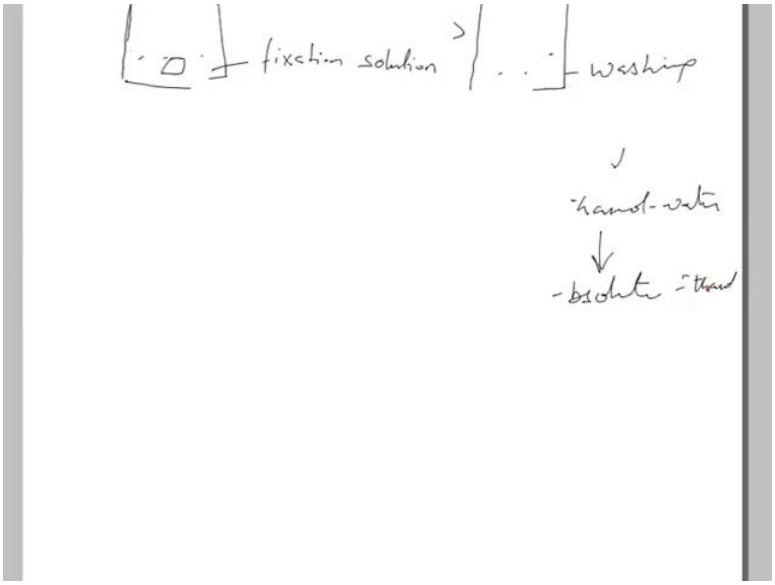

So, a series of ethanol water and finally the absolute ethanol would be utilized. Then propylene oxide will be utilised for transition. As a transition solvent, once you have done that then what we have to go about is this, particular thing has to be embedded.

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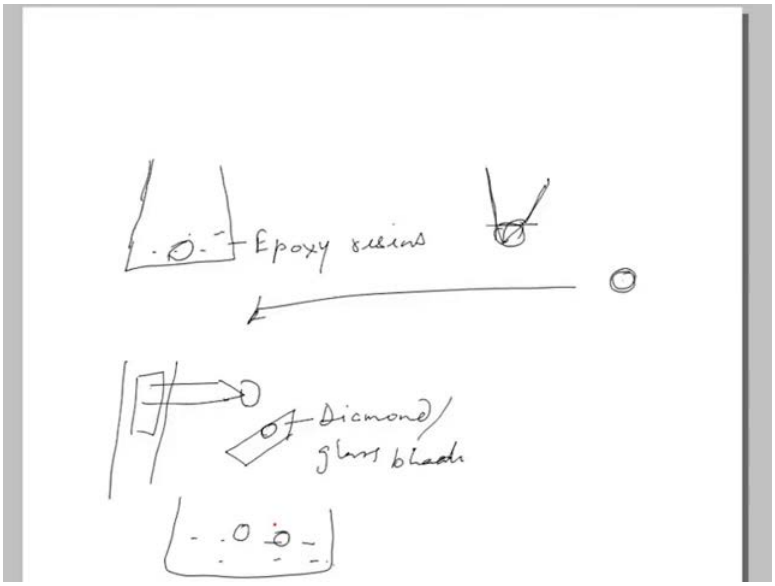

For embedding there will be the embedding solution. Here, the tissue will be kept in the embedding solution. This could be a thick section or otherwise this is what will happen. This embedding solution will be epoxy raisins.

Now this embedding solution will be then put into the oven for drying. Once it is dried then it will be taken up into append of tube. Once this is hardened this part could be cut by a scalpel or raiser. What you will get is, you will get only this part here. This will be present in here. This could be then taken to ultra microtome. The ultra microtome as we have seen earlier, it could simply be an arm. This material which is here will be put on here. There will be a blade or a diamond knife or a glass plate. It can be utilized now.

This one has a property that it will give much higher ultra thin sections. Like I said, then the simple microtome is ultra microtome because this is placed in cold conditions. It could be placed in cold conditions. Certain crave techniques has to used that we will discuss later on. Now, as this is a water tougher, the sections are cut in here. These sections will fall off onto the water trough from the edge of the knife. Then this could be picked up by the, by the specimen holder.

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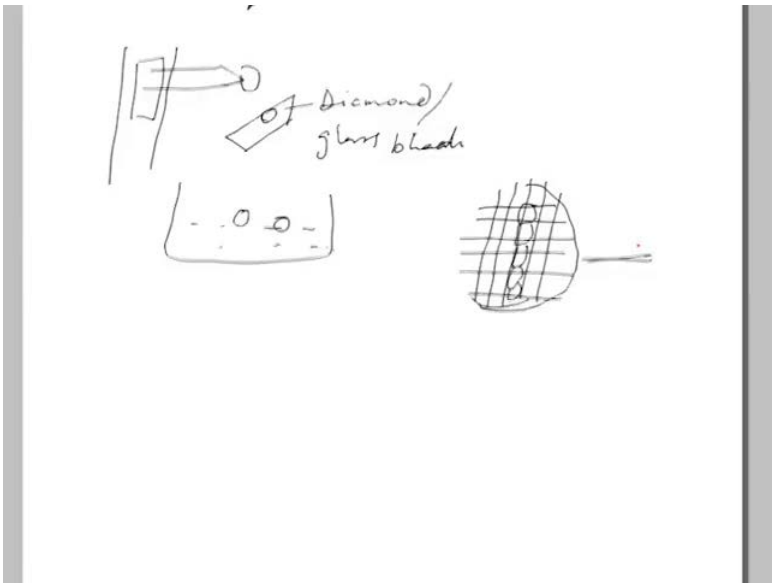

Now, this specimen holder which is a specimen grid will be a circular 3.05 millimetre grid with a mesh of copper or some other metal. It looks something like this, which has a mesh size. A specimen will be put in at this mesh and this will have a holder here. Now, this could be placed in this. Once, these are again stained in here and once these are stained and dried. Then finally rinsed with this distilled water, they could be placed into the electron microscope for view. So that is how the whole thing works.

So, I hope you have been able to understand this whole specimen preparation. It is like, you fix it then you dehydrate it. Then you make sections and finally, you stain it on the specimen grid. Then you insert it for viewing in the electron microscope. It could be side entry or top entry device. In this lecture we will stop here. In summary what we have discussed in here? It is the different components of electron microscopes. We have seen the basic differences between the light microscope and electron microscope.

You have advantages as well as disadvantages particularly you have advantage of resolution in electron microscope over light microscope. You have disadvantage of you cannot use life cells as you can do in many techniques of light microscopy. In the next lecture we will continue discussing about the various other methods to create contrast. In transmission electron microscopies as well as we are going to discuss about the scanning electron microscopy.

Thank you.