Experimental Biotechnology
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Lecture - 35
Microscopy Part - 2

Hello everybody, this is Dr. Vishal Trivedi from department of biosciences and bioengineering IIT, Guwahati. And what we were discussing? We were discussing about the microscopes and in that discussion we have started with the light microscopes and then subsequently we discuss about the fluorescence microscopes. And in the previous lecture, we have also discussed how you can be able to utilize the fluorescence microscope to study the Phagocytosis.

So, now in today's lecture, we are actually going to discuss about some more aspects related to microscopy. So, one of the basic phenomena for which the people are using the microscope is that it is actually magnifying the objects and that is how you can be able to see the 2 object distinctly using the microscopes. So, the ability of a microscope to distinctly see the 2 different objects is being measured by a property known as a resolution.

If you remember, when we were discussing about the chromatography, we have also discussed about resolutions and where we were talking about the separation of the 2 different peaks whereas in the case of microscopy, the resolution is more about the ability of a microscope to resolve the 2 different objects and the distance between these 2 objects. So let us see how the resolution is being defined.

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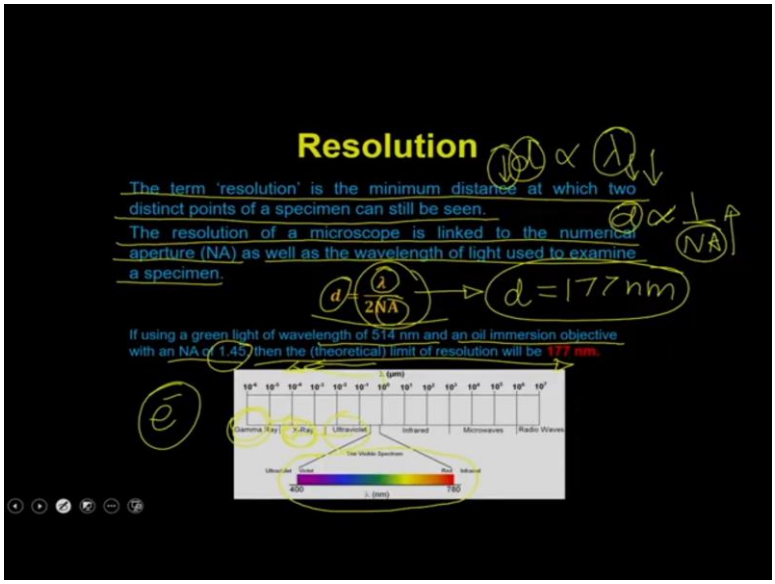

So, the term resolution is the minimum distance at which the 2 distinct point of a specimen can still be seen by the microscope. And by the definition, the resolution of a microscope is linked to the numerical aperture as well as the wavelength of the light used to examine the specimen which means the distance d is directly proportional to the lambda and inversely proportional to the numerical apertures.

Even if you use the fluorescence microscope or the light microscopes, you are going to use the visible range of the spectrum. So, if you imagine that I am using the green color light and if I use a green color light which is actually going to have a wavelength of 514 nanometer and with a light microscope where you are using the oil immersions objective, the numerical aperture is going to be 1.45.

So, if you put all these values into this formula, what you are going to get is, you are going to get the d is 177 nanometer which means with the help of the light microscope, you can be able to see the objects which are the close by to the 177 nanometer if you are using the green light as a source of light. So, there are 2 options by which you can be able to increase or you can be able to decrease this number and as you can see that d is directly proportional to lambda which means if you decrease the lambda you can be able to decrease the distances.

And remember that decrease in distance means the increase in resolution which means if I am looking at the 2 objects, which are very far away from the 1 nanometer, so if the 2 objects are 1 nanometer this difference and I could be able to see them distinctly then the resolution of

that particular microscope is going to be better than this microscope which is actually going to give you a distance of 177 nanometer.

So, decrease in d should not be interpreted as d decrease in resolution. So, decrease in d means there is an increase in resolutions, which means there are 2 ways in which you can be able to do the d is directly proportional to lambda. So, if you decrease the lambda, the d is also going to be decreased and that is how you can be able to increase the resolutions. d is inversely proportional to 1 / NA.

Which means, if I have to decrease the d I have to increase the NA. But there is a limitation until which you can be able to increase the nuclear numerical aperture of a microscope. That is why you people are not modifying the numerical aperture instead what they are doing is they are playing with the different wavelength of the light. And that is how you can be able to get the better resolutions.

So, if you see the electromagnetic spectrum of the electromagnetic spectrum, what you will see is that for the visible light, you are using that into the light microscope. And if you go from the visible light towards the ultraviolet as well as x ray and gamma rays which means if you go towards this side, you are actually going to decrease the lambda. And as you decrease the lambda, you are eventually going to increase resolutions.

The only problem is that when you decrease the lambda, you are actually going to reach to a region which is actually not going to be visibly active which means it is not going to be perceived directly by a human eye. And that is why you require an intervention so that you can be able to perceive the signals. So one of the way in which you can be able to perceive the signal which is coming either by the x rays or gamma rays is that you can actually add some x ray active or the gamma ray active molecules into the system.

So, if you want to increase the resolution, what you can do is you can actually stain the cells with molecules which are actually going to give you the signal or which are actually going to respond to these wavelengths. And that is how you can be able to visualize them, not by the naked eye, but with the help of the different types of the sensitive screens. So when you decrease the lambda, in this case, what people are doing is they are using the electron as the source of the illuminations.

And the microscopy what they are using is called as the electron microscopy. And as I said, if you see the wavelength of the electron from the web and if you are going to put that value into this formula, you can be able to calculate the theoretical possible resolution of an electron microscopy. So what I would suggest is that you should do this exercise and see how big the magnification you will get when you use the electron microscopes.

So electron microscopes are using the electron as a source to illuminate the objects and then these electrons will interact with the matter which is present within the cell. And that is how they are actually going to give you the image of that particular sample. There are 2 ways in which you can be able to process the sample and that is how you can be able to have the 2 different types of electron microscopy.

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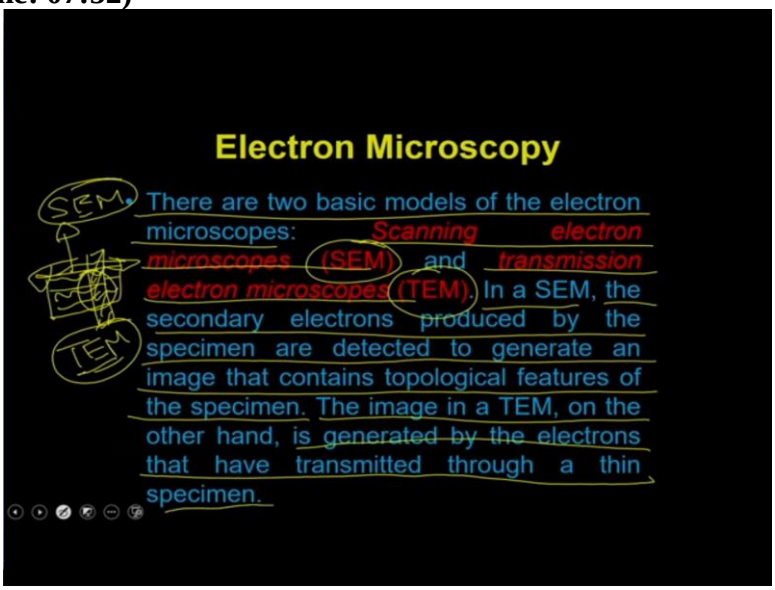

So, there are 2 basic modes of the electron microscopes, one is called as a scanning electron microscope or the SEM and other ones called as that transmission electron microscope or the TEM. So, if you have a sample, you can actually have the 2 things to observe, one how the surface of this particular object and that actually you can do simply by looking at doing with the SEM which is called as the scanning electron microscope.

So a scanning electron microscope, as the name suggest is actually going to scan the surface of that particular object. And that is how it is actually going to give you the, how the surface is look like. So, it is actually going to give you the topology, whereas if I am interested to know what is the material is and how the structures are inside, then what I can do is I can

actually cut this sample into the multiple sections and then I can be able to see all the things what is present inside and that is what is called as the transmission electron microscope.

Which means, I can actually illuminate the objects in 2 ways, in which in one way that I can just simply illuminate the top surface and that is how I can be able to monitor the surface chemistry or the surface topology. The other way is that I will just illuminate the object pass through and in that way the electron will pass through with the sample and that is how it is actually going to give me the details what is present inside the mater, sample and that is being done by the transmission electron microscopes.

So in a SEM, the secondary electrons produced by the specimen are detected to generate an image that contains the topological feature of the specimen, which means how the surface is actually that is called as the topology. So that is the detail what you get when you are going to process a sample for the scanning electron microscope. The image in a TEM on the other hand, is generated by the electron that have transmitted through a thin specimen. And that is how it is actually going to give you the details of that particular sample how the sample is from inside.

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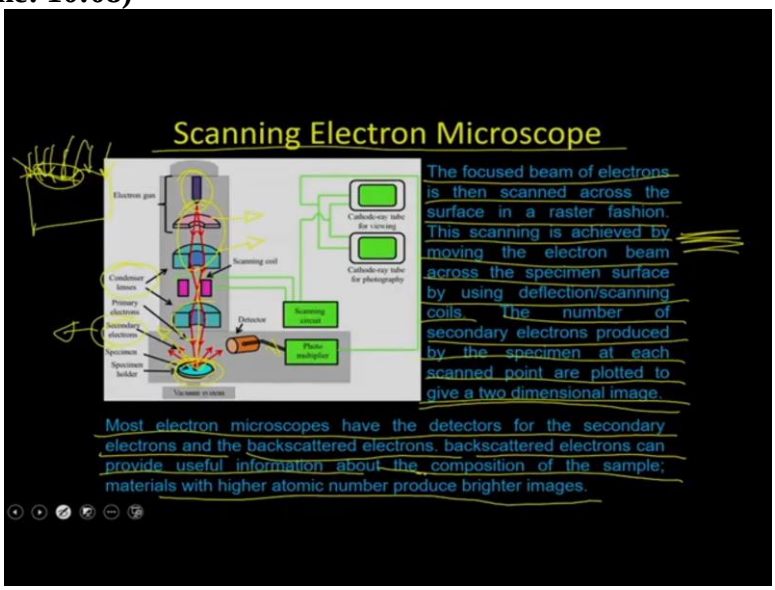

So, in a scanning electron microscope and if you have the electron gun, so you have electron gun which is actually going to generate the electrons and then it is actually going to have the magnetic coils, the job of these magnetic coil is only to focus the electrons what is coming from the electron gun and then you have a condenser lens. So, the condenser lens is actually

going to focus this and then it is actually going to allow you to bombardment the sample which is going to be placed onto a sample holder.

And once you bombardment the sample with the help of the electrons, the electrons are actually going to go into the different directions. So you are actually going to have the secondary electrons because, as I said you have to make the sample active for the electrons. So, once the electrons will go inside the electron will interact that particular molecule and then that molecule is going to generate the secondary electrons. And these secondary electrons are actually going to be detected by the detectors.

And that is how it is actually going to generate an image. So the focused beam of a electron is then scanned across the surface in a raster fashion which means it is goes like this. So it is actually going to scan the sample and this scanning is achieved by moving the electron beam across the specimen surface by using the deflecting as well as the scanning coils. The number of secondary electron produced by the specimen at the each scanned point are plotted to give you a 2 dimensional image.

Which means, if you have this is the surface what will happen is that it is actually going to bombardment the electron guns or the electronic rays everywhere and as a result, the secondary electrons are going to be produced by everywhere. But if you have a depth the electrons are going to reach to different points within that particular detector. And that is how it is actually going to give you a 3 dimensional structures or 3 dimensional information's that is how it is actually going to give you a 2 dimensional image.

Most electrons microscope has the detector for the secondary electrons and the backscattered electrons. The backscattered electrons can provide the useful information about the composition of the sample material with the higher atomic number produced with the brighter image. So, in the sample, you are actually going to generate the 2 different types of electrons one is called as the secondary electrons, other one is called as a back electron.

So back electrons are being like the beam is hitting the sample and then it is getting reflected. So in that case, the higher the atomic number, which means the higher the density of the electrons present in that particular sample, it is actually going to give you the more number of back electrons. If you analyze the data of that back electrons, you can be able to calculate the

hardness or the thickness of that particular sample or the composition of that particular sample.

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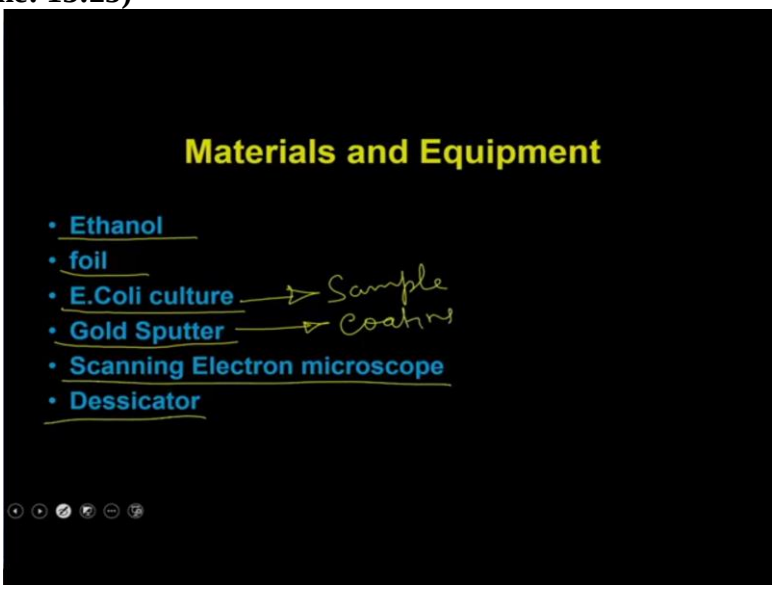

So, how to perform the scanning electron microscopes. So, for to discuss that I have taken an example of the e coli bacteria. So suppose we have to perform the scanning electron microscope of the e coli bacteria and I want to see how the bacteria look like. So material what I required is, I required ethanol, I required the aluminum foil, I required the e coli culture. So, that is the sample what we are going to process.

Then we required the gold sputters. So, gold splutter is required for the coating the sample and then you required a scanning electron microscope and as well as the dessicators. The process is having the discrete steps.

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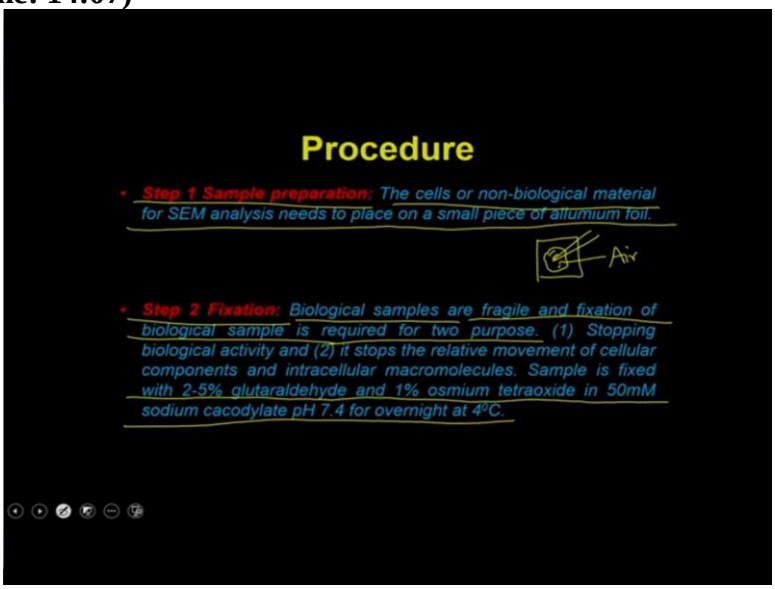

So in the step one, you are going to prepare the samples. So the cell or the non biological material for the SEM analysis need to be placed on to a small piece of aluminum foil. So what you do is you take a small piece of aluminum foil, and then with the help of the pipettes you just deposit your material onto this and then let it be air dry, so that it is actually going to make a thin film.

And then the step 2, you are going to do the fixation. So, if it a biological sample, the biological samples are fragile and it require to be fixed and the fixation required for the 2 purposes. And if you, I think remember, we have discussed how why we are doing the fixation, so fixation is only being done only to stop the biological activity also to stop the movement of the molecule within the cell.

So instead of using the normal fixative agents like methanol and acetone what we have discussed when we were discussing about the fluorescent microscope here, you are actually requiring the more highly cross linked material. And that is why you are actually going to use the fixation is done with the help of the glutaraldehyde as well as the osmium tetroxide in the sodium cacodylate buffer overnight.

So that it is actually going to do an extensive cross linking of the sample and therefore, it is actually going to give you a better fixation and compared to that what we were doing in the fluorescent microscope because there you are not doing to illuminate the sample with the electrons. So here actually there is a chances that the sample may get disintegrate when you are exposing it to a high beam of electron, because the high electrons are very high energetically very a powerful molecule. So that is how you actually have to do an extensive fixation. So once a fixation is over, then you have to do the dehydration.

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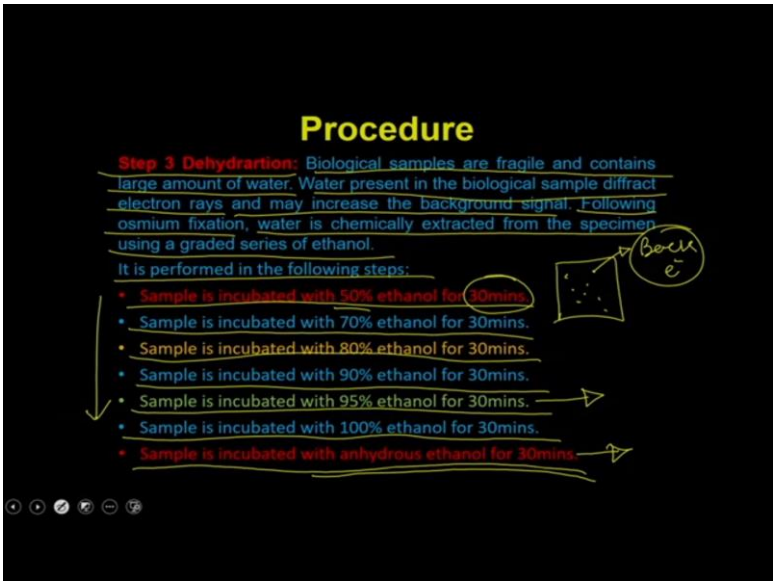

So the water what is present inside a biological sample is actually going to make the sample diffract or scattered the electron beams and that is how it is actually going to make the background more and more pronounced. So to avoid that you have to actually remove the water. So the biological samples are fragile and contains a large amount of water, the water present in the biological sample actually diffracts the electron rays and may increase the background signal.

How they will increase the background signal is that if you have a water molecule, the water molecules are actually going to increase the back electrons, when see you have the back electrons and a background of back electrons, it is actually going to make the things more hazy. And that is how it is actually going to mask the signal of your scattered electrons. And that is how it is actually has to be removed from the sample to get a very clear contrast.

So following osmium oxide fixations, the water is chemically extracted from the sample using a weighted series of ethanol. So it is performing the following steps. So what you do is you are simply soaking the sample in a different concentration of alcohol. So that and the alcohol is hygroscopic in nature, so it is actually going to start sucking the water, but you cannot do the dehydration in an abrupt manner.

So you have to do the dehydration in a very slow increment. For example, first you are going to take the sample and incubate with the 50% ethanol for 30 minutes. So that is so 50% ethanol means it is actually going to take up the water very slowly. And that is how it is actually going to start taking out the water once it is actually been reached to the equilibrium,

then you are going to incubate the sample with 70% alcohol, so you have increased alcohol further.

So that actually is going to withdraw some more liquid, then you are going to incubate the sample with 80% and then you are going to incubate the sample with 90% and then you are going to incubate the sample with 95%. So at that stage, the sample is almost there is no water present, but then what you are going to do is you are going to incubate the sample with 100% ethanol for 30 minutes.

So that actually is going to remove even if there is a minor amount of water present inside the sample. And that is how you our sample is now free of water. So sample is incubated with anhydrous alcohol, so that is the 100% or the absolute alcohol. So you are going to have the anhydrous alcohol and that is actually going to remove the last drop of water. So why we are doing the grading dehydration is because if you do abrupt dehydration.

Which means if I just take the sample and put it into the 100% alcohol first of all removal of water is not going to be smooth. It is actually going to be more from some ends and the less from the other ends. So that actually is going to change the surface chemistry and that is all actually it is going to change the structure or the morphology of that particular sample. And apart from that it, the alcohol may also damage the sample.

So when you do a graded dehydration, which means you start from the 50% and then you go slowly into multiple steps to 100% and then to the anhydrous absolute alcohol. You actually allow the sample to acclimatize to that particular environment. This means you actually allow the sample to remain morphologically intact.

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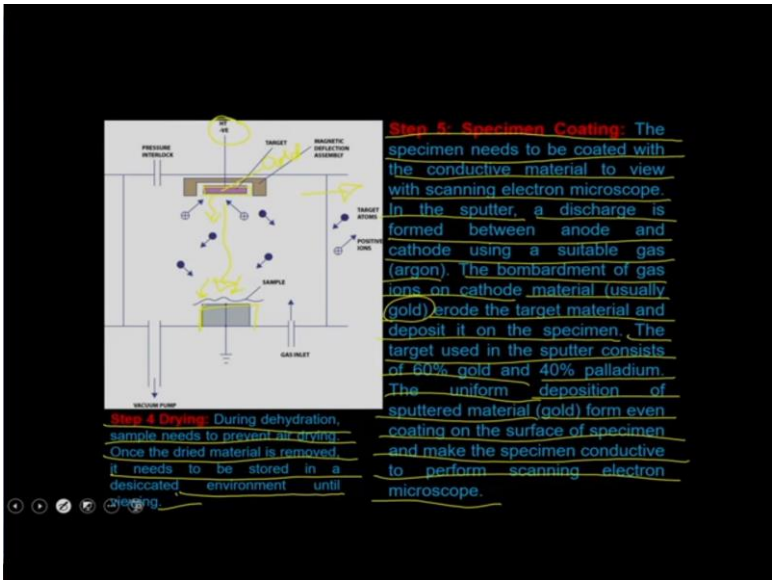

So, after that you have to do a drying step. So in Step 5, you have to do a drying so during dehydration sample needs to prevent air drying. Once the dried sample is removed, it needs to be stored in a desiccated environment, until the viewing. So this means once your sample is been dehydrated you have to do a drying under the vacuum and then you have to keep the sample under the vacuum so that it should not capture the moisture from the atmosphere.

Which means, because your sample is now dehydrated it is actually having a tendency to capture the moisture from the atmosphere. And that is why you have to do a drying step and then after the drying is over, then you have to keep this sample in desiccators. And desiccator if you do not know about the desiccator, desiccated is a box kind of thing where you actually can generate a very high vacuum like 10 to power -5 or 10 to power -7 vacuums.

And then that vacuum is actually going to make the system inert. So it will remove all the air. So that is how there will be no moisture present in that particular chamber and that is why your sample is going to be preserved. Now you have to keep that sample under the desiccated conditions until you are not ready to analyze the samples. So just before the analysis you have to do the step 5.

So step 5 is the specimen coating. The specimen needs to be coated with the conductive material to view with the scanning electron microscope. So, as I said you have to add something so that or you have to stain the sample in such a way so that it should be respond to the electron beams. So, in this case, you are actually going to coat the sample because you

are just simply interested to see how the surface morphology is look like. So and that it is what the purpose is scanning electron microscopy.

So, that you do with the help of a sputter. So in a sputter a discharge is formed between a anode and a cathode using a suitable gas. So, this is a gold sputter, what you have is you have a target, which means this is actually having the gold and that is actually being present at a negative and then you are, this is under the vacuum. So under the vacuum you are actually keeping your sample on to this block and then you are asking and then you are actually doing a bombardment of the gas ions.

So when you are doing the bombardment of gas ions, what will happen is it is actually taking up the gold, and it is actually showering the gold on top of this sample and that is how it is actually going to make a smooth one layer of the gold coating onto the sample. So, the bombardment of the gas ions on to the cathode material, usually the gold, erode the target material and deposit it on to the specimen.

So, when you do a bombardment of your target substance like the gold in this case, with the ions, the material will come out which means the gold is going to come out and then it is actually going to deposit on to the sample and it is actually going to deposit as per the morphology of that particular sample. So, it is not going to disturb the morphology of the sample, instead it is just going to make a steam coating.

So that you can be able to study the morphology of that particular sample because wherever the gold is present, it is actually going to diffract and it is actually going to scattered the electron and that is how you can be able to capture the back electron as well as the scattered electrons. The target used in the sputter consists of 60% gold and the 40% palladium. The uniform deposition of the sputtered material that is the gold forms even coating on the surface of the specimen.

And make the specimen conductive to perform the scanning electron microscope. So, once you are done with this coating of the sample, then your sample is, you do not need to keep the sample under the vacuum and then you can just load this sample into the scanning electron microscope and then you can be able to visualize with using the electron microscope.

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And then when you visualize, you will look like this. So, what you see is this is actually the bacteria and what you see is the surface of the bacteria is like this, because we have done some treatment and that is how all the bacteria what you see is actually having a change in the surface chemistry and what you see is some of the in formations like this is the scale through which this photo has been taken.

So the scale is 2 micron meter, the magnification what you see is 3.63k and this is been collected in the secondary electron mode, you can actually have the other kind of mode as well, which actually gives us some more information about the surface chemistry of the molecules. So now from here we will move on to the transmission electron microscope and we will discuss about how you can be able to use the transmission electron microscope to answer the some of the biological questions.

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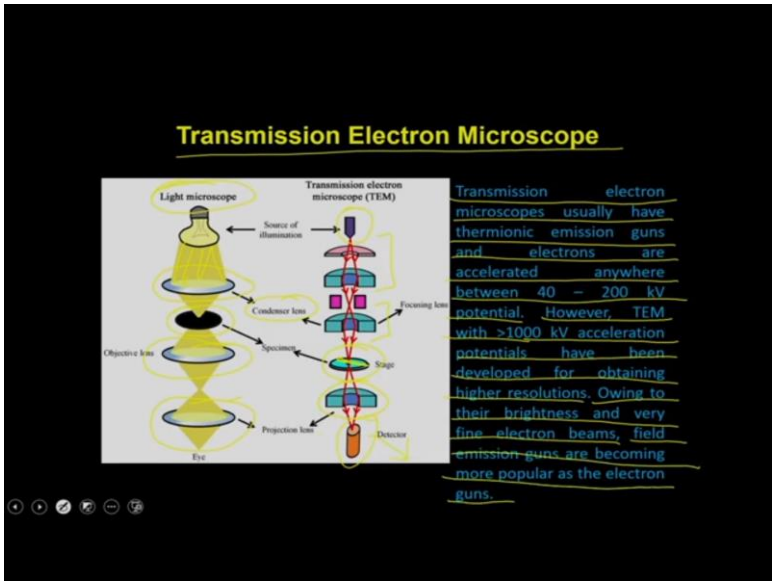

So transmission electron microscope is very simple as it is like a light microscope except that you are using the electronic beam as a source of illumination instead of the light. So I have given you a comparison. So in this case you have a light microscope. So light microscope is using a bulb and that is actually sending the light and then it is being focused by a condenser lens and then it is being illuminated the objects.

And then once it is illuminated the object you can actually capture the image with the help of the objective lenses and then you can be able to see that with the help of the eyepiece. Similarly, in the case of transmission electron microscope, you have the electronic gun through which the electrons are coming, and then you have the magnetic coils through which you can be able to focus these electrons.

And then you have the focusing lens and that focusing lens is actually going to focus the beams and then it is actually going to eliminate the sample which is going to be kept onto the stage and then whatever the electrons are coming out from the sample are going to be focused by the projection lens and then it is actually going to go on to a detector. So in this case, you are actually going to have a detector which is sensitive for the electrons compared to that here you can simply use the eyes to perceive the signal.

So then you can be able to get the same information. So here also the lights are passing through the sample and that is how you are actually getting the details of the cell as well as what is present inside the cell. Similarly, in the case of transmission electron microscope also you will get the details of inside the cell. So the transmission electron microscope usually

have the thermionic emission guns and the electrons are accelerated anywhere between 40 to 200 kilo volts potentials.

However, TEM with more than 1000 kilo volt acceleration potential have been developed for obtaining higher resolutions. Owing to their brightness and a very fine electron beam field emission guns are becoming more popular as the electron guns.

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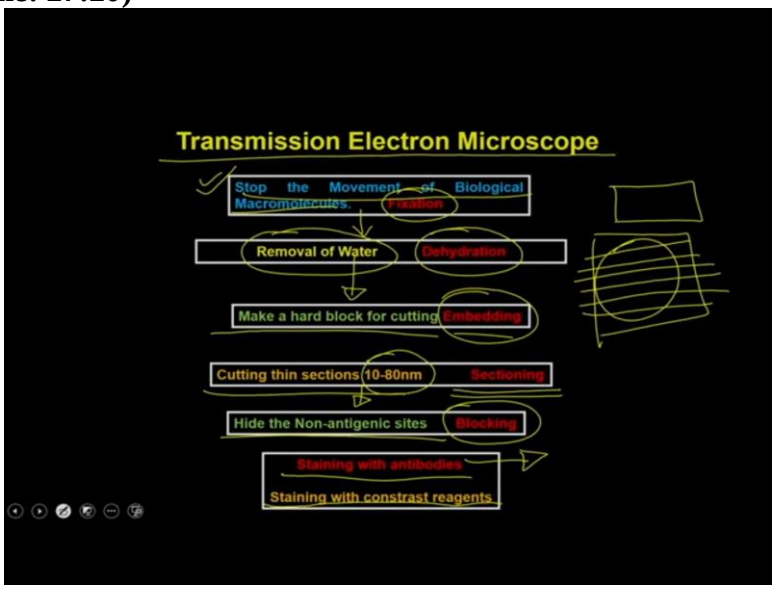

Now, when you do a transmission electron microscope the processing of the sample is little different what you have done for the scanning electron microscope and you have to do additional step because now you want the sample to, electrons to pass through the sample and then actually it is going to give you the image. So, what you have to do is the first step is same that you have to stop the movement of the biological macromolecule which means, you have to do a fixation step.

After the fixation the second step is also same that you have to remove the water so that you have to do a dehydration, so that the water also should not interfere in the increasing the background because the water is going to interact with the electrons and it is going to diffract these electrons and that how you are actually going to have the heavy background. So, you have to remove the water.

After that, since you have to make the, suppose this is your cell or this is your actually cell. So, it is actually not possible to eliminate a cell and then you can be able to look at what you have to do is you have to cut this cell into multiple pieces. And that how the single layer of

that particular object has to be illuminate with the electron because these electrons are high energy electron but they are not that high that you can be able to illuminate the thick object and that actually will pass through with that.

So, that is how you have to make the thin slices of that, so that you will illuminate and then you will be able to collect the electrons what is coming through the object and that is how actually you can be able to reconstruct the images. So, for that you have to make a hard block for cutting and that is been done with the help of a process called as the embedding. Once you are done with the embedding, which means you have put this into a block then you can cut the section so you can cut a section of it, 10 to 80 nanometer.

And that you will do with the sections. Once you are done with the sectioning, then the next step is that you have to hide the non antigenic side, which means you have to do a blocking step and then you have to do a staining with the antibody which means you have to do a staining with the primary as well as the secondary antibodies. Remember that this is a transmission electron microscope.

So compared to the fluorescent microscopy, here you have to use a secondary antibody which is actually coupled or labeled with the heavy metal so that it is actually going to respond or it is going to interact with the electrons, what you are going to use to illuminate the objects. Then you have to stain with the contrast reagents, so that you will be able to see better contrast of the sample. And that is how you are actually going to prepare your sample and you can be able to observe the cells with the help of the transmission electron microscope for more details and that actually details are going to be inside the cell.

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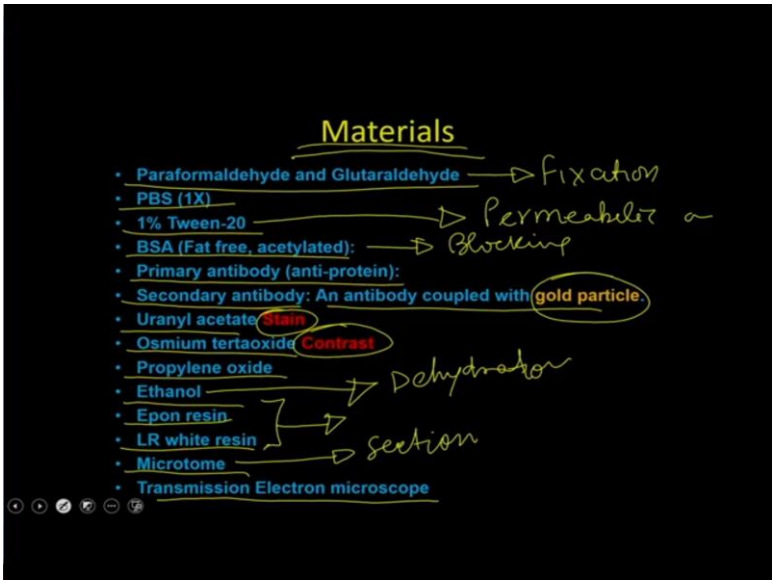

So, these are the material what you require, if you want to do a transmission electron microscope, you require a paraformaldehyde and a glutaraldehyde. So that is you require for fixation of the sample. Then you require the PBS which is generally for the washing, then you require the triton x 100 that is for the permeabilizations, then you require the BSA that is for the blocking.

So when you prepare the BSA exactly the same way as what we have discussed when we were discussing about the fluorescent microscope, that you have to filter that with the 0.54 micron filter, then you require the primary antibody which is going to be against your antigen and then you require the secondary antibodies or antibody which is coupled with the gold particle.

So that you will be able to see the gold particles under the microscope and that is how you it is actually going to give you the image of that particular sample. You require an uranyl acetate which is actually going to be a stain, then you require the osmium tetroxide, which is actually going to be a contrast agents, then you require the propylene oxide, ethanol which is going to be for the dehydration step.

Then you require the different types of resins like epon resin or the LR white resins. So that is for the embedding. And this is then you require the microtome which is for cutting the sections. So you require a microtome and then you require a transmission electron microscope, so that you will be able to illuminate the objects. And you can be able to observe the objects in the transmission electron microscope.

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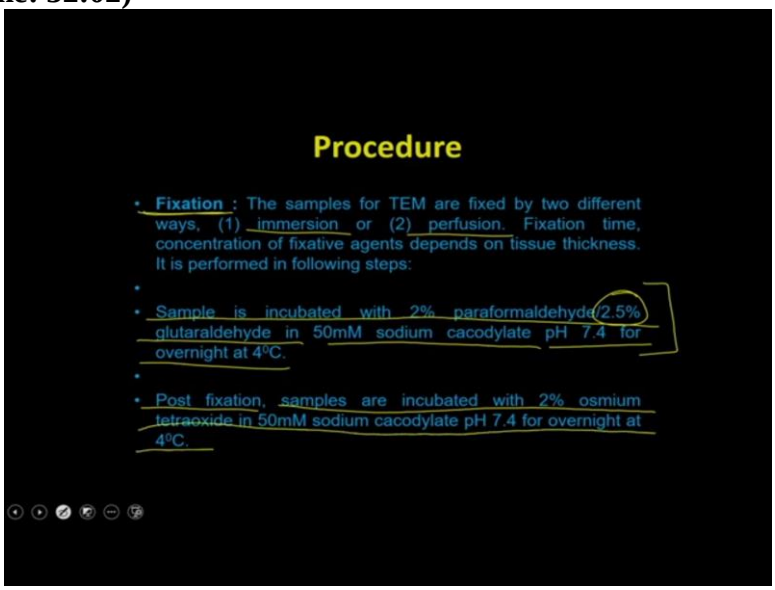

So in the step one, you are going to do a fixation. So fixation is same, remain the same as we before but you have to do some more, agents you have to use the different agents compared to what we have done. So fixation can be done in 2 ways either you have to do immersion or the perfusions. So sample is incubated with 2% paraformaldehyde and 2.5% glutaraldehyde mixture in 50 milliliters, sodium cacodylate ph 7.44 overnight.

So that actually is going to give you the extensive cross linking of the protein what is present inside the sample, and that is actually going to give you very high fixations. Post fixations, the samples are incubated with the 2% osmium tetroxide, in 50 millimeters sodium cacodylate buffers for overnight at 4 degrees Celsius. Then step 3, you are going to have the dehydration. So, the addition step is same as before that your biological samples are fragile.

So, you have to do sequential dehydration that we have discussed when we were discussing about the same. So, you just have to follow the same kind of dehydration steps and that actually is going to remove the, all the water what is present in the samples.

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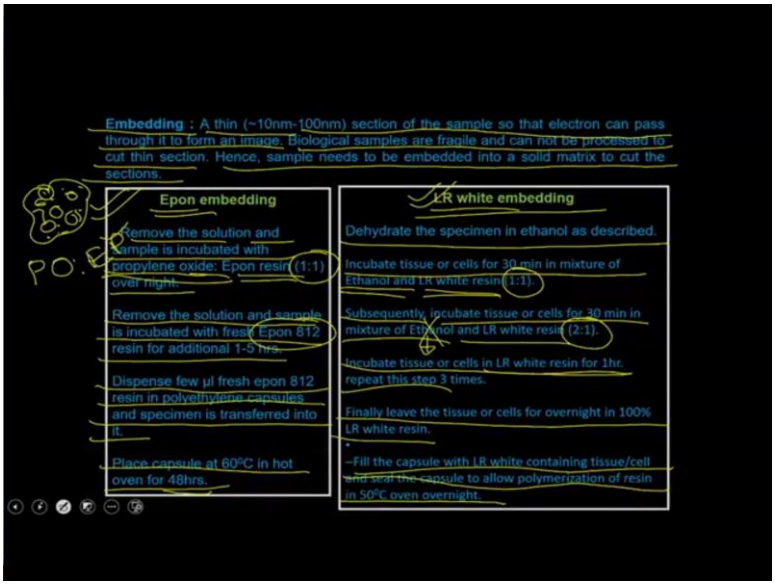

After that, you have to do an embedding. So, a thin 10 nanometer to 100 nanometer section of the sample you have to cut so that the electron can pass through it to form an image. Biological samples are fragile and cannot be processed to cut thin sections. Hence sample need to be embedded in a solid matrix to cut the sections. You have the 2 different types of matrix which you can use, you can use the epon matrix or you can use the LR white embedding.

The process of both the embedding is different. So, in the case of epon embedding, what you have to do is first you have to remove the solutions like the solution what you are using for dehydrations and then the sample is incubated with a mixture of the propylene oxide and epon resin 1 is to 1 mixture overnight. Once that is done, then you can remove this solutions and the sample is incubated with the fresh epon 812 resin for additional 1 to 5 hours.

And so what you see here is you are actually adding the propylene oxide and epon resin mixture. Now, what we have done very slowly we have removed the propylene oxide and then we have added the epon resins and that actually is now been changed. Now what you do is dispense the few microliter fresh epon resin in polypropylene capsules and the specimen is transferred into it. Place the capsule at 60 degree in hot oven for 48 hours.

So, what you are exactly doing is, because you have to do a embedding only not from outside from inside also. So what you are doing is you are simply replacing the solvent what is present inside the sample with the resin. So, you can imagine that if I have a cell and you have a mitochondria, you have a chloroplast, you have all this kind of thing and then you

have a nucleus, all these material is now been replaced by a alcohol because you were doing the dehydration steps.

So, what you do is you are just simply putting this into a mixture of polypropylene oxide and a epon resin. So, what happened is all this alcohol is now been replaced and it is going to be replaced by the epon. So, that you are actually providing the strength to the sample not from outside but from inside as well. So, once that is done, then you are actually going to keep the epon resin into a capsule. And then after putting some resin then you put your sample and then again you put the resin on top.

And then you keep this capsule into a 60 degree for overnight and that, or the 48 hours. And that actually is going to make this sample matrix. And that is how it is actually going to be ready for cutting the sections. Apart from that you can also do the LR white embeddings. So, in that case, you dehydrate the sample in ethanol as we discussed before, and then you incubate the tissue or the cell for 30 minute in a mixture of ethanol and the LR white 1 is to 1 mixture.

So, the process remains the same. In this case, we were using the propylene oxide and epon resin, here you are using the ethanol and LR white resins 1 is to 1 mixture. Subsequently you incubate a tissue or cell for 30 minutes in a mixture of ethanol and LR white resins in a mixture of 2 is to 1, which means you have reduced the concentration of the alcohol and you are increasing the concentration of LR white.

Now, in the third step you again do the same thing and then now this time you have removed the alcohol you have put only into the LR white resins and finally leave the tissue for overnight in 100% LR white resins. And then you have to do again the same thing you fill the capsule with the LR white containing tissue and seal the capsule to allow the polymerization of resin in 50 degrees for overnight in ovens. So, the process remains the same except that you have 2 different types of matrices what you can use to prepare the sample for cutting the sections.

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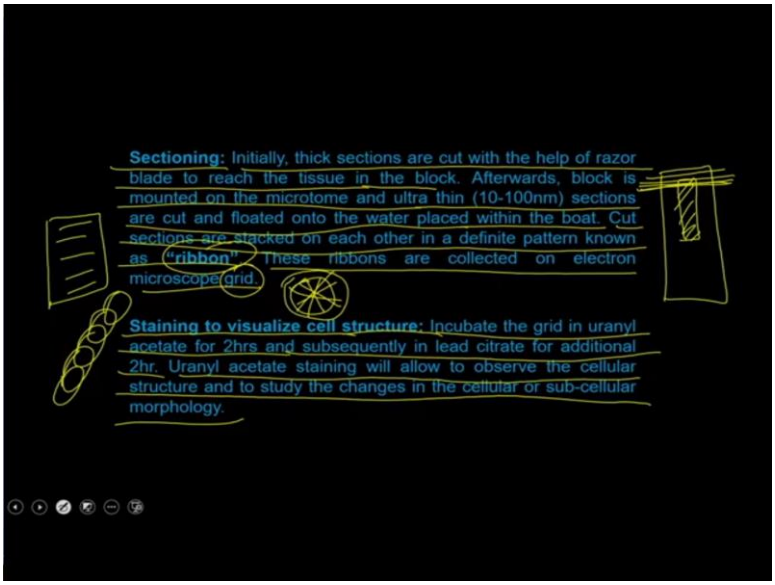

After that you have to do a sectioning. So, initially the thick sections are cut with the help of a razor blade to reach the tissue in a block. So what you have is, you have, going to have a block of the LR white or the epon resins and here you are going to have a sample. So these are, this is the sample what you have. So what you are going to do is initially you are going to use a razor, cut the samples in a thick sections like the 10 micron or 1 micron sections.

So when you do, going to cut the 1 micron sections, you are actually going to reach to these particular tissues. Once you reach to tissue the block is mounted onto a microtome and ultra thin sections are cut and floated onto a water placed with the boat and you cut the sections are stacked onto each other in a different pattern known as the ribbon. So, what happened is when you are cutting these sections, they are actually coming into a boat like structures and this boat is filled with the water.

So, what happened is these sections are coming like this in a, like a ribbon kind of situations and then what you do is exactly these ribbons are collected on to a electron microscope grid. So this electron microscope grids are the circular grids where you have the grid like pattern and on this grid you can be able to just collect all these sections together and then these grids can be mounted into the transmission electron microscopes.

Then you have to also to do a stain to visualize the cellular structures. So you can advance your sample are present onto the grid then you can incubate the grid in a uranyl acetate for 2 hours and subsequently in a lead citrate for additional 2 hours. So uranyl acetate will stain will allow to observe the cellular structures and to study the changes in the cellular or the sub

cellular morphology. So if you stain the cells with the uranyl acetate, it is actually going to allow you to see all different types of organelles like mitochondria, golgi bodies, endoplasmic reticulum, nucleus and all that.

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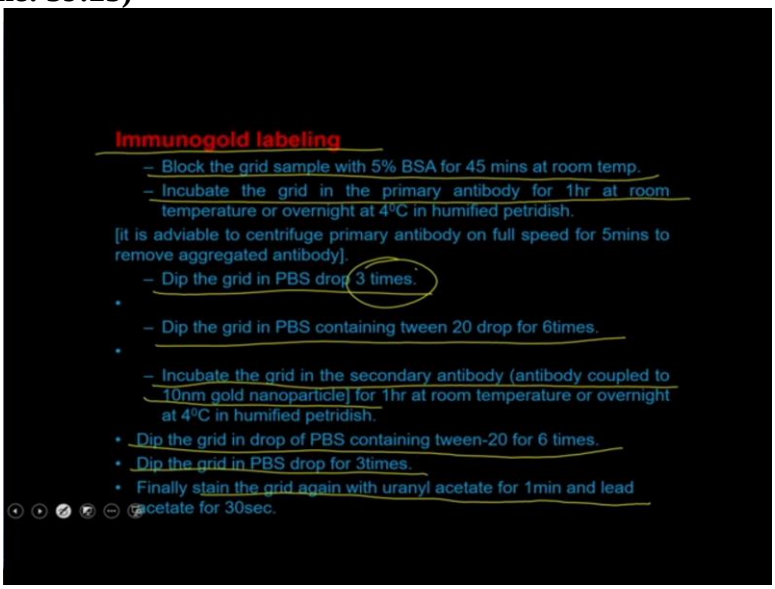

Then you have to do the immunogold labeling. So what you do is you block the grid with the help of the BSA, the incubate the grid in 5 primary antibodies, and then you do a washing to remove the unbound antibodies. So you dip the grid in a PBS for 3 times. And that is actually going to remove the unbound pro antibodies. And then you actually dip the grid in PBS containing tween 20.

And that is actually going to give you even the additional washing of the antibodies and that is how you are going to remove all the nonspecific antibodies. And then you incubate it with the secondary antibodies. Secondary antibodies are going to coupled to a 10 nanometer gold nanoparticles. So you have the multiple options either you can use antibody which is coupled to a 10 nanometer gold particles or to a 5 nanometer gold particle. So, you have the choices because you can be able to very clearly see these nanoparticles.

And that is how you can be able to very pinpointly say that this is the location of the particular protein. And then you have to do a washing exactly the same way that you have to do first washing with the PBS and then PBS containing the tween. Finally you stain the grids again with the uranyl acetate, so that you, whatever the uranyl acetate has been lost while you are doing this immuno staining is again come back and it is actually going to give you the morphology of the different organelles.

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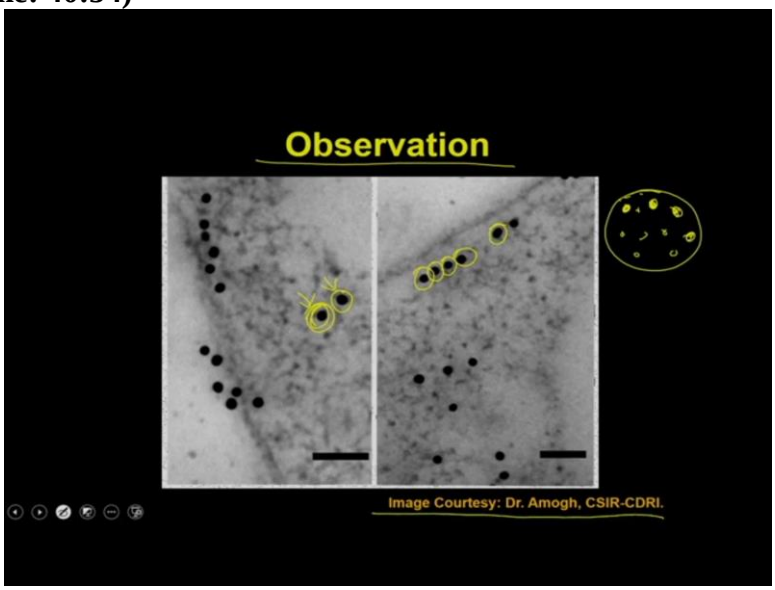

And then you are actually going to put this or you are going to charge the grid into the transmission electron microscope and what will happen is once you insert that into the microscope, it is actually going to be illuminated by the electronic gun and that for the electron will pass through and that how wherever the electron will not pass through, for example, this is your sample and these are the places where you have the gold.

So, what will happen is that when you illuminate this object to all other places, the electrons are going to be passed through whereas, the electron will not be able to pass through from these locations where you have the gold particles or to the heavy particles and because of that, it is actually going to give you a black colored dots. And that is how you can be able to know that these are the locations of my proteins.

So this is the typical image what you will get when you do a TEM image, TEM analysis. So what you will see is you are not going to see the very fancy membrane and all that. What you are going to see is you are going to see a dots like this and these are the dots which are actually been formed when the 10 nanometer gold coupled antibody was binding to a primary antibody.

And so, these are the locations of the protein of your interest which are in the cell. If you want to see you can see these cells with the help of the internal state staining and that is how it is actually going to tell you whether these dots are present in the mitochondria or golgi

bodies or endoplasmic reticulum or nucleus and all that. This image is being provided by the, one of my friend in CDRI, Dr Amogh.

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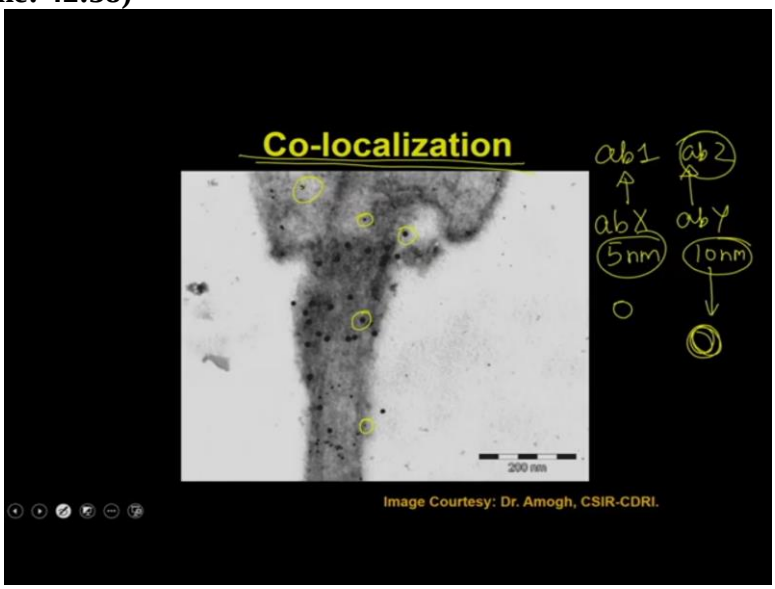

Now, if you want to use the transmission electron microscope, you can be able to use it even for the co localization studies. So, if you remember when we were discussing about the fluorescent microscope, I said that you can be able to use 2 different set of secondary antibodies to localize the protein a and protein b and you can be able to ask the questions whether the protein a and protein b are co localizing to each other or not, whether they are localization is different and all that.

So, in this case also you can be able to do that kind of experiments where you can do the 2 antibodies you can take the antibody 1, you can take the antibody 2 and similarly, you can take the secondary antibodies like x and y and what you have to do is the x you can take the 5 nanometer gold labeled and in this case you can take the 10 nanometer gold. So, what will happen is wherever the ab2 is present, it is actually going to give you a circle of the 10 nanometer.

Wherever the ab1 is present it is actually going to give you a circle of 5 nanometer. For example, in this case you see these are actually the 5 nanometer circles and these are the 10 nanometer circles. So, in some places what you see is that for example, here you will see that the 10 nanometer as well as the 5 nanometer are actually, are sticking to each other, which means, at this place the protein ab1 and ab2 are interacting with each other, which means they are actually co localizing and probably forming a complex.

Whereas the other places the 10 nanometer as well as the 5 nanometer signal is different. So, that is how you can be able to very precisely be able to say that whether the protein a and protein b is interacting with each other or not, and whether they are localizing to a same compartment or not. So, this is all about the different types of microscopy techniques. So, far what we have discussed we have discussed about the light microscopy, we have discussed about the fluorescent microscopy.

And then in today's lecture we have discussed about the scanning as well as the transmission electron microscopy, we have also understood how to process the sample for all these microscopy's. So in our subsequent lecture, we are going to discuss few of the experiments related to these microscopy techniques. And then we also going to discuss some more aspects related to the cell biological tools. So with this I would like to conclude my lecture here. Thank you.