Environmental Chemistry and Microbiology
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Module - 8
Lecture - 43
Microscopy - II

All right, welcome everyone. This is the last lecture in module 8 and the last part of Microscopy. This is lecture 43.

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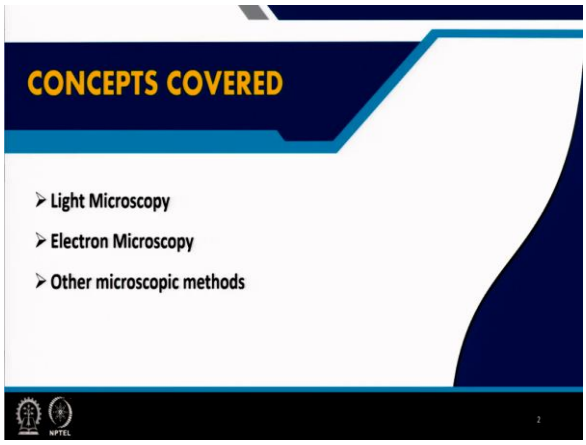

So, as I mentioned in the previous lecture, we are going to be covering the remaining part of light microscopy as well as electron microscopy and a few other recent developments in microscopic methods.

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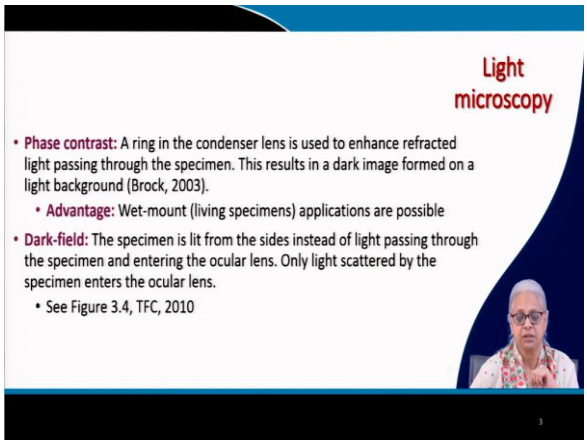

So, let us continue with light microscopy. Here I have 2 different modifications; one is phase contrast and the other is dark-field. Here is a simple schematic that shows us the light path in bright field microscopy, in phase contrast and dark-field microscopes.

So, we will start with phase contrast. So, in phase contrast, we have our light source; and there is an annular diaphragm right in front of the light source. What it does is, it forces a fraction of the light to pass through the annular diaphragm and then through the condenser lens. This light is then brought to focus on the specimen. Now, depending on the organelles in the sample, each of these organelles is going to have small differences in refractive indices and because of that, the path length is going to be altered. So, the direction of the light is going to be altered, which is shown with red arrows. The light that is transmitted right through the specimen that is unaltered, is shown in blue. So, the refracted light as well as the transmitted light is going to be passing through the objective lens. It will then pass through what is called a diffraction plate and then it will reach the ocular lens and the eye. What happens in this case, like I said is, we are utilizing the differences in refractive indices of the different parts of the cell to create an image. And this results in greater differentiation of the internal structures. And the biggest thing that is pointed out here is that the outer covering or the external covering or pellicle of the cell is clearly visible, which is in contrast to bright field. Because, in bright field, you have a light pellicle against a light background. So, it is not very clear. In this case, you have a dark background and a light-coloured pellicle. So, this outer, external covering of the cell is basically much clearer in phase contrast microscopy. I think, one of the other textbooks mentions very clearly that one of the advantages of phase

contrast is that wet mount applications are possible. So, you do not have to have dry mount which in some cases can distort the features of the cell.

Then we come to dark-field. In dark-field, the specimen is lit from the sides. Now, I have mentioned in bright field that the light is passing through the condenser lens; it is brought to focus on the specimen; it is being transmitted through the specimen. We are dealing with bacteria or other microorganisms and wherever light is not able to pass because of the presence of certain organelles, that is seen as dark. So, these organelles are dark and all the others are light, because light is either reflected or passing through. So, these are the things that happen in bright field. Now, here in dark-field, an opaque disk is placed in front of the light source. So, whatever is going by the side of the opaque disk, is being brought to focus using a condenser lens on the specimen. So, here we have the specimen. So, some part of the light will be lost. It is scattered. And that is shown by the red arrows. The blue light is what is passing through the specimen. That is collected by the objective lens and to the ocular lens and then the eye. This results in a very peculiar image, you have a dark-field. It is a dark-field microscope, so you have a dark background and you are illuminating the object by reflected light. So, it is a part of the entire light that is coming. It is only the reflected light that is caught and is used to generate this image. So, this is basically what you get.

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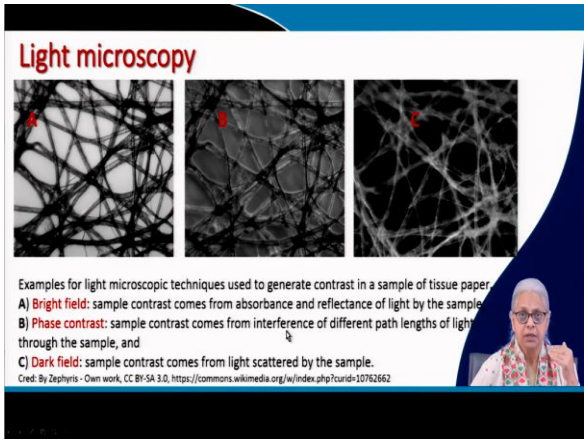

So, these are outputs of these types of microscopes. So, the first one; this is a sample of tissue paper. You have a light background and light is passing through this specimen. So, you know that either light will pass through the specimen, that is transmitted light; or any material that is in the water sample is going to either reflect, scatter or absorb (light). So, when it (the

material or object) is dark, the darker it is, it means, it is absorbing all the wavelengths. Reflected light is where you see colour. So, whatever colour you see is the reflected colour. And scattered is basically not going to be of much use, except in dark-field microscopy. So, that is where you are looking at the scattered light or the light reflected from the sides of the specimen. So, here you have bright field, phase contrast. You can see in this case, the background is not completely dark. Here, it is slightly greyish black.

So, there are all kinds of images on the internet. There are light backgrounds, dark backgrounds, black backgrounds; all of that is there. And this is mainly because of differences in the refractive indices of the different materials and the interference of light path. So, you can refer to the textbook for more detail. It is explained much better. And then we have dark-field. So, dark-field, like I said, is reflected light. And that is what you get against a dark background.

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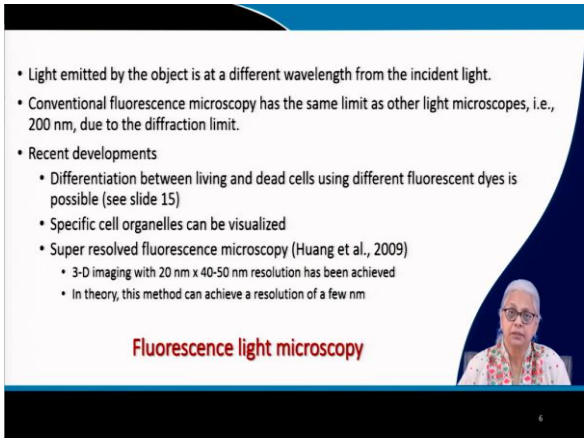

All right, so we now come to a new development in light microscopy; and that is fluorescence microscopy. I need to remind everyone that fluorescence is a phenomenon- where the light emitted by the object is at a different wavelength compared to the incident light. So, some of you may be familiar with the natural fluorescence of chlorophyll. Now, we know that under sunlight conditions, if any cell has chlorophyll in it, it looks green. We are all familiar with algae and so on. In our environments, including plants, all of them have a green colour because of the presence of chlorophyll molecules. Now, when I see an object as green, it means that the light is; light can do various things; it can be reflected; it can be transmitted; it can be absorbed; it can be scattered. When I see a particular colour, it means

that wavelength, the wavelength that is coming to my eye is reflected light. So, when I see something as green, red or yellow, then that is the colour that is being reflected back into my eye. All other colours are either being absorbed or transmitted through the sample.

When I say that chlorophyll is naturally fluorescent, one of the most important experiments that many of you may be familiar with is to put this chlorophyll under UV light. Now, UV light has a wavelength below 300 nanometers. Under UV light, chlorophyll does not look green, it looks red. And that is because of this fluorescence of chlorophyll. So, the same phenomenon can be used to visualise all other types of cells, which do not have naturally fluorescent compounds like chlorophyll. So, we can use fluorescent dyes.

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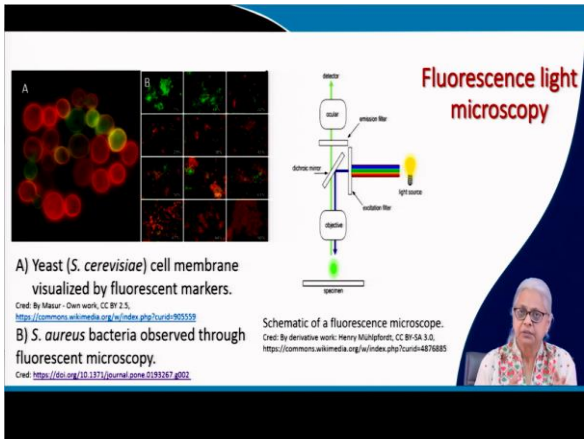

And this is what we see over here. This is from a paper. Here you see yeast cells. These yeast cells have been dyed by fluorescent markers or dyes. And they have attached themselves to the cell membranes. Now, these cell membranes have different colours and so on. So, it is much easier to look at the cell using a fluorescence microscope. Therefore, you can, like I said, it is much easier to see particular parts of the cell. In this case, it is the cell membrane. In the second case, in B; this is from a particular paper recently published I think. This is *Staphylococcus aureus* that has been observed using fluorescence microscopy and more than one dye. So, here you see a series of images, which have been taken; after the cells have been dyed with 3 different fluorescent dyes; and each one of them will react with the cell membrane. Now, if the cell membrane is intact, it means the cell is likely to be alive and viable and capable of reproducing. On the other hand, if the cell membrane has been damaged, then the reaction to the fluorescent dye is going to be different. The colour that is

caught in this case is red. In case of the living cells or intact cell membranes, the colour is green and that is because of different dyes reacting in a different manner. So, this kind of difference in the reaction of different cells to these dyes can be used to separate living cells from dead cells. In this particular paper, they have used image processing software to enumerate cells under these different conditions. That is the number of, I think dead cells. Yes. I think it is the number of dead cells that are shown in these series of images, where the green cells are living cells and the red cells are dead cells.

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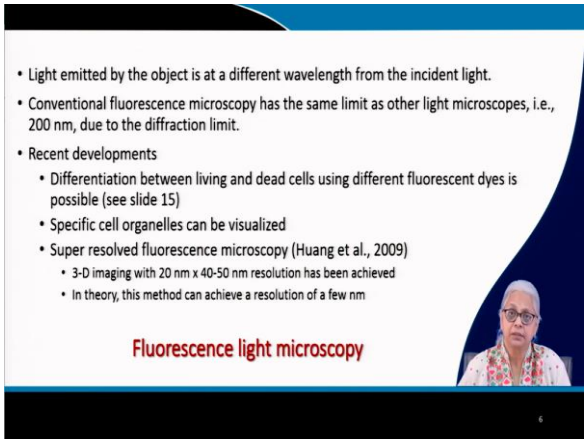

So, that is a major advancement you might say, because one of the biggest limitations in microscopy is that we did not have the ability to differentiate between living and dead cells. Now, if we are able to use this kind of method and differentiate between living and dead cells, then it becomes very easy; and we do not have to go for plate counts and so on. As I said, we can also visualize specific cell organelles.

Then we come to another major advancement. So, I think I mentioned in my introductory lecture that super resolved fluorescence microscopy was given a Nobel Prize, I think in 2014. One of the reasons is that super resolved fluorescence microscopy has allowed us to image certain structures within the cells, resulting in 3D images where the resolution has gone down by 10 times.

So, instead of the limit of 200 nanometers, now the limit is 20 nanometers; and people are working on, pushing the limit even further to maybe 1 or 2 nanometers. So, this was unimaginable with light microscopy. So, it is a huge advancement, like I said, in light

microscopy. And the current results which I am going to show you in a little bit is, 3D images that have been achieved with a resolution of 20 nanometers by 40 to 50 nanometers.

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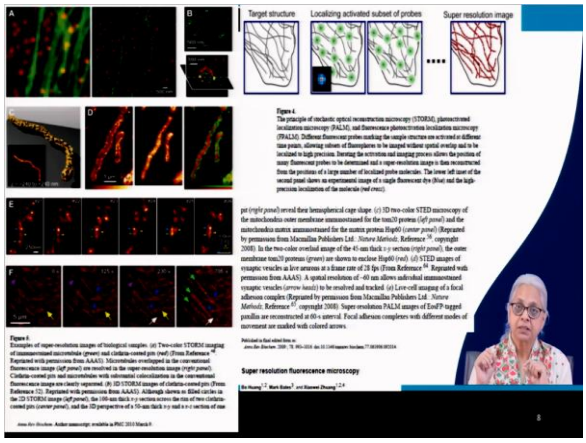

So, the one that I am going to focus on in this particular case is C. C is a mitochondrial membrane. And since you know a little bit about mitochondria; so, it may have some relevance. So, this is a 3D image of a mitochondrial outer membrane that has been stained by a particular protein. So, this protein has fluorescence properties. Therefore, it can be imaged under a fluorescence microscope. And you can see the detail. It is around 240 nanometers. The basic principle of this super-resolved microscopy; or in this case, the stochastic optical reconstruction microscopy and so on. The idea is that you have fluorescent dyes which attach themselves to particular cell organelles. So, you are not looking at the entirety of the cell, which we saw in dark-field, bright field and phase contrast. In this case, we are looking at only a particular cell organelle.

So, if you have a structure like this, you are going to be able to attach the fluorescent dye only to these fibres. And that is what is shown over here. And the end result is an image like this. So, you do not have an image of the entire structure under the microscope, but you have enhanced images of only specific organelles that are part of the cell. In any case, it is an important development. So, that is about fluorescence light microscopy. Let us move to another method.

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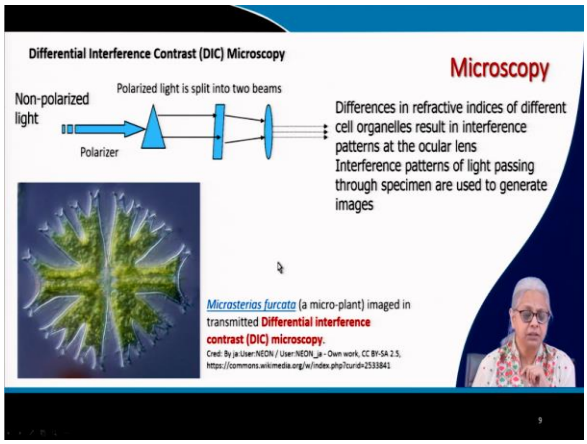

The next method is called differential interference contrast microscopy or DIC. In this case, non-polarized light is passed through a polarizer. The polarizer will split the light into 2 beams. Those are passed through 2 prisms and what reaches the ocular lens, again because of interference patterns that are generated by differences in refractive indices of the different cell organelles, that will create a particular image. And these interference patterns of light will be used to generate these images. And this is one of the outcomes. So, this is a micro-plant (microalgae) cell.

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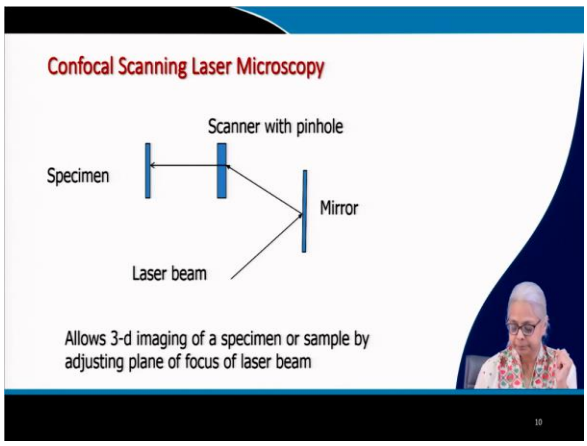

Then we come to another modification which is also becoming very popular; and that is confocal scanning laser microscopy. So, here we have a laser beam. It is reflected off a mirror. This beam, after reflection of a mirror, passes through a scanner which has a pinhole, and then it is incident on the specimen. Now, if you generate a series of images, by changing

the plane of focus; this plane of focus is going to be changed again and again. That will allow a series of images to be formed, which can then be assembled together to create a 3D image of the specimen.

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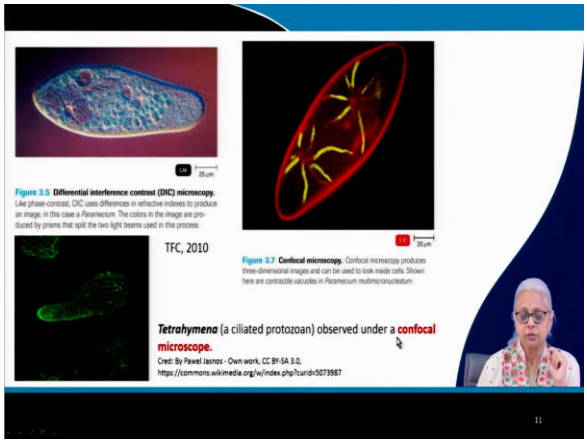

So, here are some examples of both DIC microscopes and confocal microscope. So, just like phase contrast; the 2 are similar. Phase contrast and DIC use differences in refractive indices of the different cell organelles to produce an image. This is a paramecium. We have already seen several bright field images of paramecium. This is a DIC image of a paramecium.

Then we have confocal microscope images. So, here are 3D images again of a paramecium. And enhanced organelle is these contractile vacuoles within the cell. Another one that is shown over here is a ciliated protozoa. You know that paramecium is also a ciliated protozoan and this is a different one. This is *Tetrahymena sp.* which has cilia. So, it is the cilia that have been dyed by a fluorescent dye and under confocal microscopy, you can see the cilia very clearly.

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Here is another output from confocal laser scanning microscopes. And here I think, again they are either fluorescent dyes or some other image enhancing method. So, these days, what you have is a lot of image processing software that can be used to enhance different parts of the image that is generated either by light microscopy or even electron microscopy. So, here we have diatoms. These diatoms have different organelles.

So, we have the cell wall. The cell wall is cyan-coloured. So, cyan is a bright blue. So, this fluorescent blue is the cyan colour, and that is the cell wall. The red colour is associated with the chloroplasts. There is blue DNA, which is a little hard to see. But in this particular case, you can see some blue material at the centre of the cell; and that is the DNA. Then you have the green membranes. So, here you have the green membranes and you have several other organelles that are part of these diatoms.

I have already mentioned that diatoms are a major group of algae which are found in oceans and waterways. They are considered to contribute to the amount of oxygen in the atmosphere; they are major contributors and so on.

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That is all about fluorescence light microscopy. And these are some of the images that we have been able to get with the fluorescence microscope in our lab. So, here we have S*taphylococcus*, which is a Gram-positive bacterium. It was isolated from groundwater. And you can see, because of the dark purple; it is not black, it looks black, but it is really a dark purple coloured bacterium because of the gram staining method. So, you can see very clearly, it is coccoid and it is gram positive, because of the dark purple colour. And this is from groundwater.

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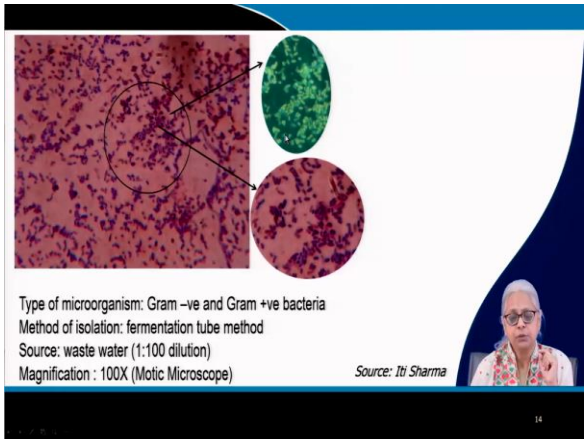

Here we have a mixture of gram positive and gram negative cells. And I have already mentioned to you that the staining procedure results in purple cells for gram positive bacteria and red cells for gram negative bacteria. Now, when you have wastewater; wastewater has thousands of different types of cells. And under the light microscope; so, this is a bright field

image. Under the bright field image, in this, you can see, most of the cells are purple and some of the cells are pink; but it is very hard to see them. Under the filter; so, this fluorescence microscope has many options. It has got a bright field option; it has got a dark-field option and a fluorescence option. Now, in fluorescence microscopy, we also use something which I did not talk about and that is a filter.

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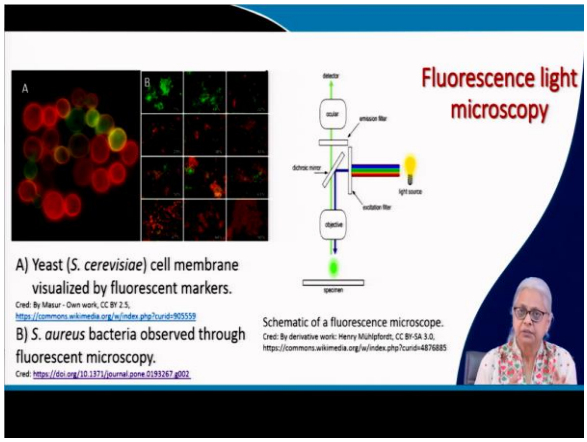

Now, this filter is the excitation filter, which allows only certain wavelengths of light to pass through. This light is incident on the object. And then, the wavelength that is emitted by the object is a different wavelength. So, that goes to the dichroic mirror and then through the emission filter and the ocular lens and finally, to the detector. So, this is what you are looking at. We have specific filters and specific dyes, and the combination of the filter and the dye will result in different colours.

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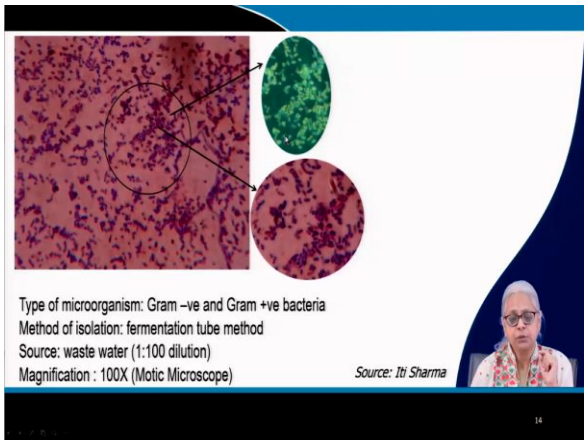

So, coming back to this. We can see that the colours are a little more obvious. Here, purple and pink are not so easy to differentiate. Pink is so light in comparison to purple, that everything looks more or less purple. So, here you can see, there are yellow-orange colours and a faded green colour. So, you can see the same image through the fluorescence option in the microscope will give you this kind of image which is sometimes better. So, if you have a software for enumerating the bacteria, it makes it easier to differentiate the colour and therefore the images and enumerate the bacteria on that basis.

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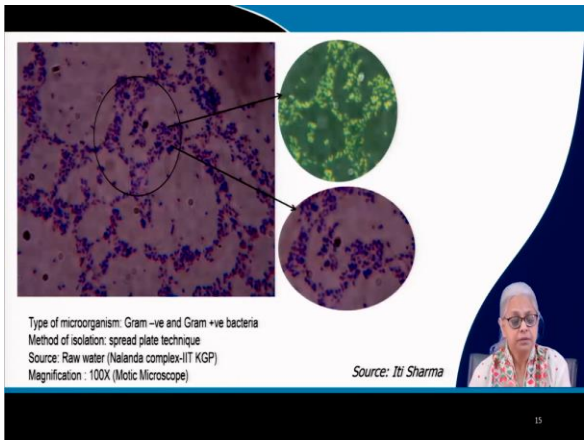

This is a raw water sample. Again, the same thing. It has a mixture of gram positive and gram negative bacteria. The method of isolation was a spread plate. The same thing is shown over here. It actually has a mixture of purple and pink cells. But you can see, purple is much

darker compared to pink. So, purple dominates under bright field. It is very; I would say, no one would probably want to count the pink cells. They are there but they are hard to see. Through the filter, in the fluorescence option, you can see the different colours. So, you have green and orange cells. And they are much easier to distinguish.

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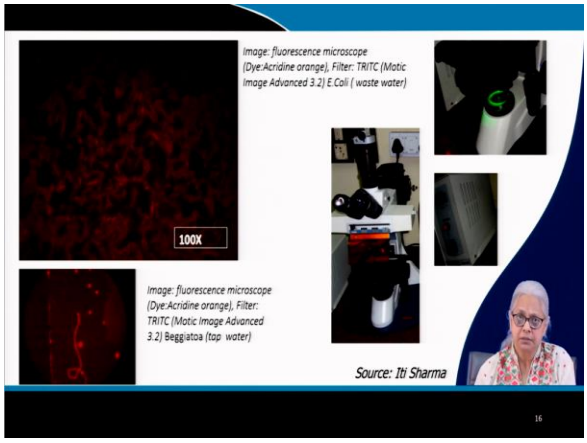

So, here we have more images using fluorescence microscopy. We have limited ourselves up until now to acridine orange. So, in this particular case, we have acridine orange. We have a filter called TRITC. And like I said, a filter is part of the fluorescence phenomenon, in fluorescence microscopes. It is the combination of the dye and the filter which results in an image. You can see the wastewater sample and *E. coli.* You can see most of it is *E. coli.* Now, I should also make it a point to say over here that you cannot really identify the bacteria in a particular sample unless it is already isolated and then visualised under the microscope. So, most likely, the wastewater contains large numbers of *E. coli*, but it needs to be verified using biochemical tests. Here is another bacterium and that is *Beggiatoa,* taken from a tap water sample. The dye and the filter remain the same.

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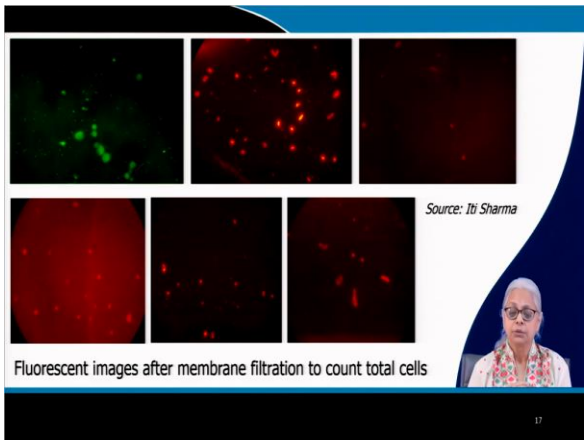

More images; so, we have fluorescence images. You can see green fluorescence. Now, I also need to make another point over here. When we use acridine orange; acridine orange is a fluorescent dye, but it fades very, very quickly, especially when there is stray light in the room. So, all this work; the fluorescence microscopy work has to be done in a dark room, pitch dark, so that the fluorescent dye does not lose its fluorescence, because it is a very short half-life for these fluorescent dyes.

Now, this green image is after the dye has kind of faded away. This is just acridine orange, no other dye. The orange and yellow colours are associated with the dye at the time that it is created. So, the very first 5 minutes is when you can do some useful work with this dye. And that is why, having a camera and a PC is very important, because, then you can capture the images in the very first 5 minutes; and then count the number of bacteria when you get time.

So, this is useful for enumerating bacteria and for storing images on either a PC or any other device. So, this has taken a lot of the tedium out of enumeration of bacteria. So, you cannot distinguish between living and dead cells. So, I should make it a point to say that green and yellow over here are not living and dead cells, it is just the fading of acridine orange.

Then we come to another major advancement in microscopy; and that is the use of electron microscopes.

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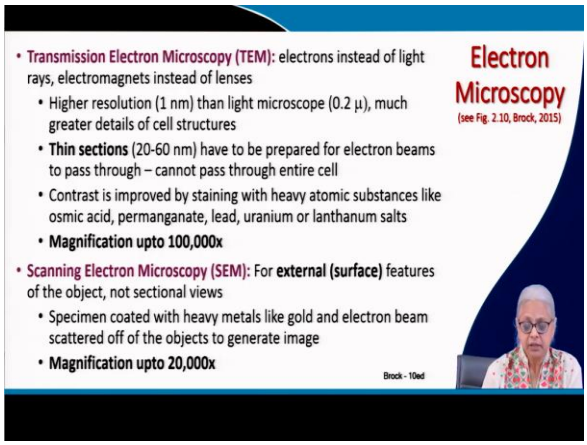

Now, within electron microscopy, we have 2 major types of microscopes; one is scanning electron microscopes and the other is transmission electron microscopes. Now, in both, electrons are used instead of light rays; and electromagnets are used instead of lenses. Now, we are not visualizing images in electron microscopy in the same way as we do in light microscopes.

So, in light microscopes, we are actually looking at the image; and whether we do it through an ocular lens or we do it through a camera; either way, it is the actual visual image. In electron microscopes, you are not really looking at the object; you are generating an image utilizing this principle of electrons as light rays and electromagnets as lenses. Then you generate an image which is again through image processing software and that is how you visualize the specimen. Transmission electron microscopes have very high resolution. It goes down to 1 nanometer in comparison to light microscopes, which have a normal detection limit of 0.2 microns. So, this level of resolution will allow you to see greater detail of cell structures.

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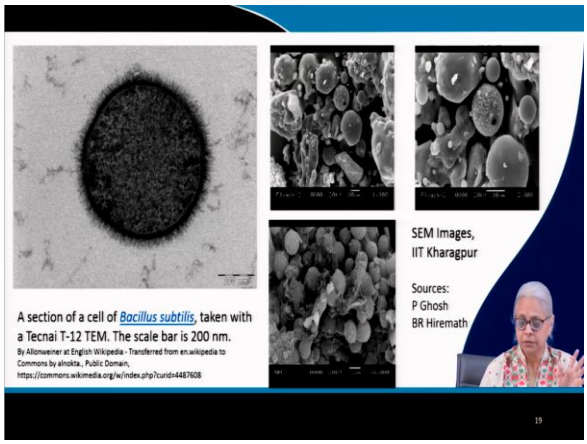

So, here you have a cell. This is *Bacillus subtilis*. And it has; you can see the bar; it is 200 nanometers. So, you can see. These are the fimbriae, most likely, these are the fimbriae around the cell. The cell wall is visible; the plasma membrane is visible; and so is the entire cytoplasm. So, you can see that the DNA is most probably distributed throughout the cytoplasm.

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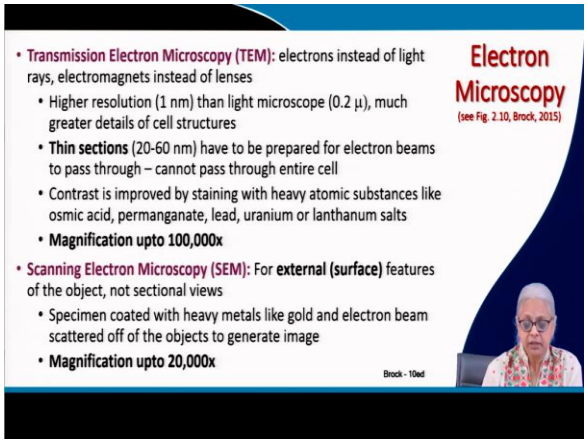

Thin sections of 20 to 60 nanometers have to be prepared for the electron beams to pass through. They cannot pass through an entire cell. And for creating the sections, you need fair amount of preparation. Preparation of the sample is done by providing staining with heavy atomic substances like osmium, permanganate, lead, uranium or lanthanum salts. So, this will help in; first thing you do is stain the section and then improve the contrast and so on.

So, like I said, the magnification is around hundred thousand times. In scanning electron microscope, you do not get the same level of detail; you do not have a sectional view; you can only visualize the external features of the object. And you again need specimen preparation or sample preparation. The specimen has to be coated with heavy metals like gold; and the electron beam is scattered off this surface to generate an image. The magnification that we have been able to get is up to 20,000 times.

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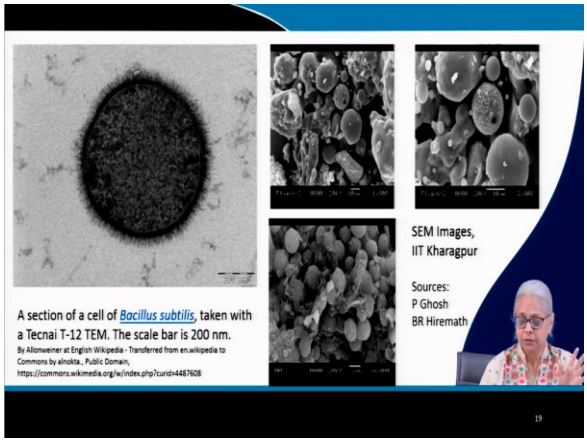

So, here we have some images from our lab. Here we have fly ash particles. Fly ash particles are basically the particles that are released after coal is burnt in thermal power plants; and it comes out through the flue gases. And when these particles settle, this is what we get. This is the fly ash we get after these particles settle back to the ground.

They are generally spherical. I think the degree of sphericity is associated with the degree of combustion. So, the greater the combustion of the coal, the more spherical the particle is likely to be. So, these are 10 microns. This is the scale. 10 microns; and this is magnification in the optical range. This is about 1000 times; and the second one is 2000 times. You can see, this is the centre of the image that has been enhanced in the second case.

In the next image, what you see is SEM images. And it is very difficult to understand. Let me say two things over here. One is the magnification; 6000 times magnification; and the scale is 2 microns. So, each of these spheres, they can be coccoid bacteria, because it is the same

range in terms of size; or they can be granules of compost. This is a compost sample. So, it can be either.

Now, you can understand why simply using electron microscopy is not sufficient for identifying an unknown object. You need several methods to verify whether the particular object that is under the microscope is actually what you think it may be. So, like I said, this may be coccoid bacteria or it may be granules of compost; or any other thing as well.

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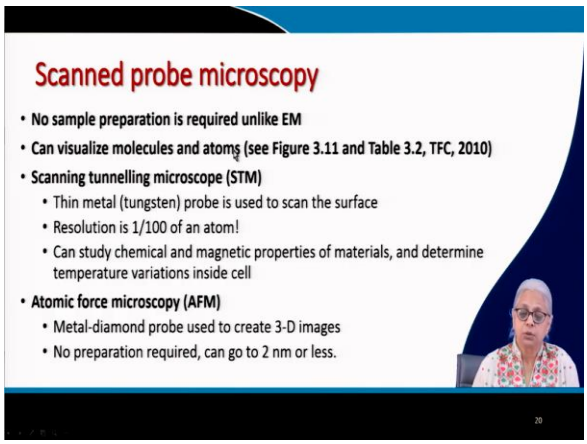

Then we come to another type of microscopy and that is scanned probe microscopy. Within that, we have two options. We have scanning tunnelling microscopes and atomic force microscopes. So, the biggest benefit of these types of microscopes are that no sample preparation is required, unlike electron microscopes. You can visualize molecules and atoms very easily. I do not have these images. I can add these images later. And then, we come to scanning tunnelling microscopes, STM for short, where we use a thin metal probe to scan the surface.

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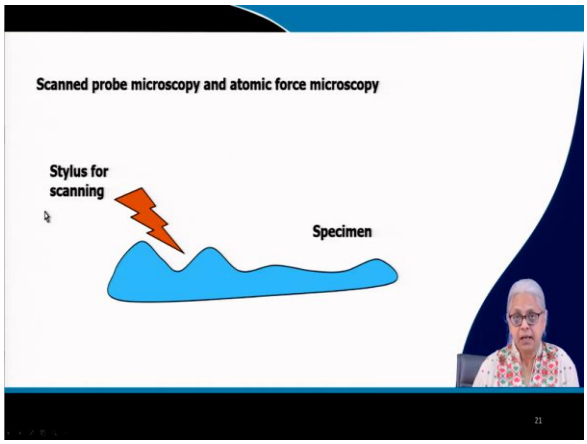

So, we have a stylus type metal probe. And this metal probe will move as the surface of the specimen varies. So, the height of the specimen, the surface of the specimen will vary and the stylus will move along with that surface. So, the same principle is applied in both types of microscopy. In scanning tunnelling microscopes, a thin metal called; may be tungsten that is used to scan the surface. The resolution that they are claiming is one hundredth of an atom. So, one hundredth of an angstrom is what is possible with scanning tunnelling microscopes. This can allow you to study chemical and magnetic properties of materials as well as temperature variations within the cell. With atomic force microscopes, we have a metal diamond probe that is used to generate 3D images. Again, no preparation is required and we can go down to a level of 2 nanometers or even less than that.

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These are some outputs from atomic force microscopy. So, we have different cells, *Lactobacillus rhamnosus*, *Lactococcus lactis*, *Lactobacillus plantarum*; all these are different types of species which have been visualized using atomic force microscopes. Now, it is important for me to say something over here. That if you have a pure culture which has been defined as a pure culture, either based on plate counts or biochemical testing and so on, then you can use these pure cultures and follow it up with atomic force or scanned probe microscopy and then get an idea of what the individual cells of that particular species looks like. So, it takes a combination of 2 or 3 methods to understand more about any particular species. So, these are atomic force images. Then we have a fluorescence microscope image and another correlative AFM image of a macrophage.

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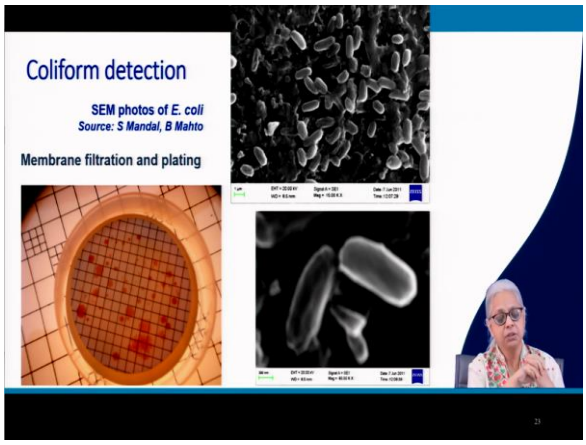

These are other outputs from our lab. So, we have membrane filtration with plating. This is under a magnifying; simple magnifying glass will give you an idea of the size of the colonies. If you have a chromogenic media, you can differentiate different types of species, based on the colour of the colonies and the texture, size, shape of the colonies. All these things can be done. And then we have SEM images. So, in the first case, we have *E. coli*. This has a magnification of 15,000 times. So, the images are pretty clear. You can see individual cells. You can see their shape, their size and texture. All these things are visible under 15,000 magnification. You can go all the way to 80,000; but in this case, the image is slightly blurred. In either case, these are SEM images.

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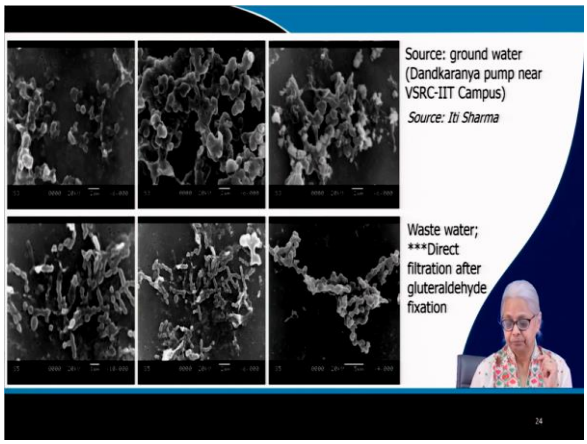

Then we have 2 other sets of samples. We have groundwater and wastewater samples. You can see that when you are dealing with natural water samples, you have a huge amount of mix of species as well as organic matter from various other sources. So, it becomes very difficult to differentiate between microorganisms and the organic matrix that may be part of the sample.

So, you can see in the groundwater sample, there is a lot of organic material as well as inorganic material and it is very difficult to make sense out of these images. Now, in wastewater, because the bacterial population is so much higher, you can see these repeating images. These repeating images are most likely to be microbes, which has to be confirmed by other tests. And you can see; based on the size, based on the fact that you have repeating images of similar types of objects, so that is what tells us to a great extent that these are microbes that are present in wastewater.

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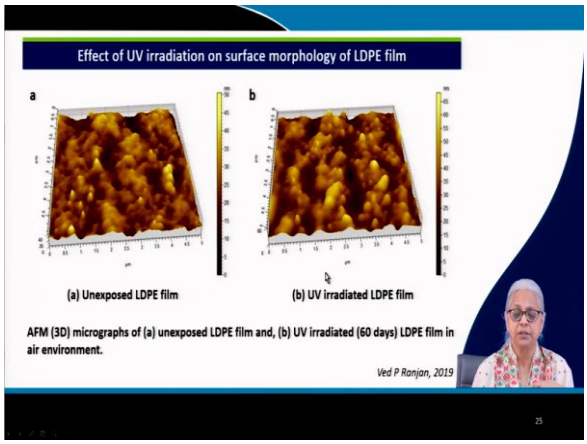

Here are images that have nothing to do with microbiology, but our outputs from our lab, just to show you what is possible with atomic force microscopes. So, this is a low-density polyethylene film and in the x, y direction, scale is in microns. But in the z direction or the elevation or height of the sample, we have nanometers. So, you can measure the roughness of a film or any other object.

So, you can measure the height. So, what you are getting is 3D information about the object. So, this is the original film; and this is after exposure to UV light. You can see how the roughness has increased because of degradation of the film.

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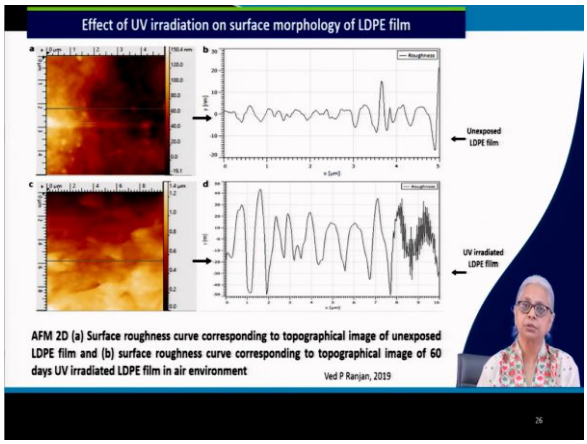

And this roughness; you can take a sectional view of the change in elevation of the film. So, in this case, a sectional view at this point has been taken and the roughness has been measured for the original film and compared with the UV-(ir)radiated film. So, you can see the level of roughness in nanometers. And that, these are some of the things that can be done with atomic force microscopy.

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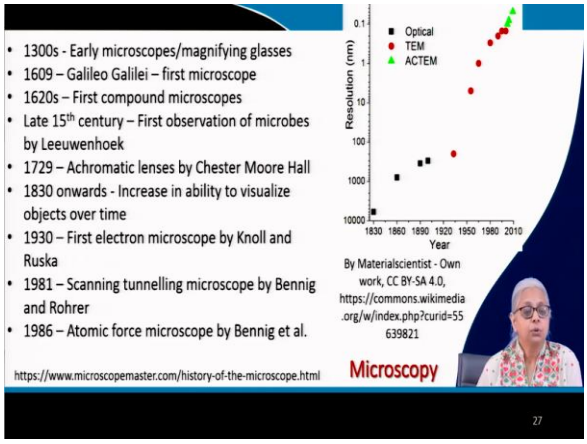

Then, I am going to end this particular lecture with a little bit about the history of microscopy, because it is obviously a very important part of microbiology. Because, without microscopes, we would not be able to understand or visualize the microorganisms that we are all interested in. So, the earliest microscopes and magnifying glasses, they were more like magnifying glasses. They are as old as the fourteenth century or in the thirteen hundreds.

And the first microscope was invented by Galileo; and that was in 1609. The first compound microscope was in 1620. In the late fifteenth century, the first observation of microbes was made by Van Leeuwenhoek. Then we have 1729, when achromatic lenses were invented by Chester Moore and Hall. Then, 1830 onwards, there has been a huge increment in the use of compound light microscopes and our ability to visualise objects using these microscopes.

Then comes 1930 when the first electron microscope was invented by Knoll and Ruska. In 1981, the scanning tunnelling microscope was invented by Binnig and Rohrer. And in 1986, atomic force microscopes were invented by the same group of researchers or scientists. Over here, in the top right corner, what you see is resolution over time. By now, you are all clear about the fact that improving the resolution of microscopes is the main objective in

microscopy. And we have now reached a level that is far below 1 angstrom. So, we can now visualise molecular structures; we can visualise DNA, molecules, proteins; all of these can now be visualised very easily. The resolution of optical microscopes, as I have already said, under fluorescence microscopy, it has now reached around 20 nanometers. So, now we are somewhere in this region with optical microscopes. It is not limited to 200 nanometers. That is what you are seeing over here is about 200 nanometers. But we are now at the level of 20 nanometers with light microscopes. With transmission electron microscopes, we can go to about 0.2 nanometers; but with aberration correction transmission electron microscopes, it is far below 1 angstrom. So, 0.1 nanometer is 1 angstrom; and we can go beyond that.

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I will end it at this point. Thank you.