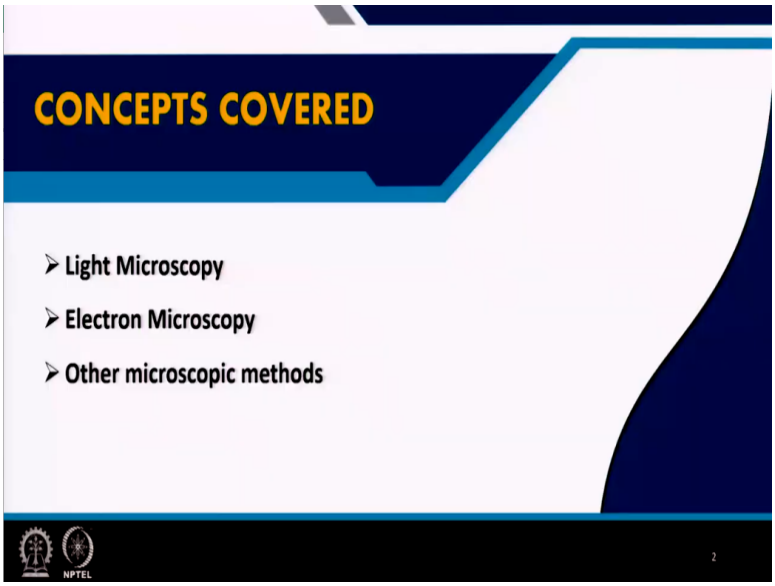
Environmental Chemistry and Microbiology

Dr. Anjali Pal
Dr. Sudha Goel
Department of Civil Engineering
Indian Institute of Technology - Kharagpur

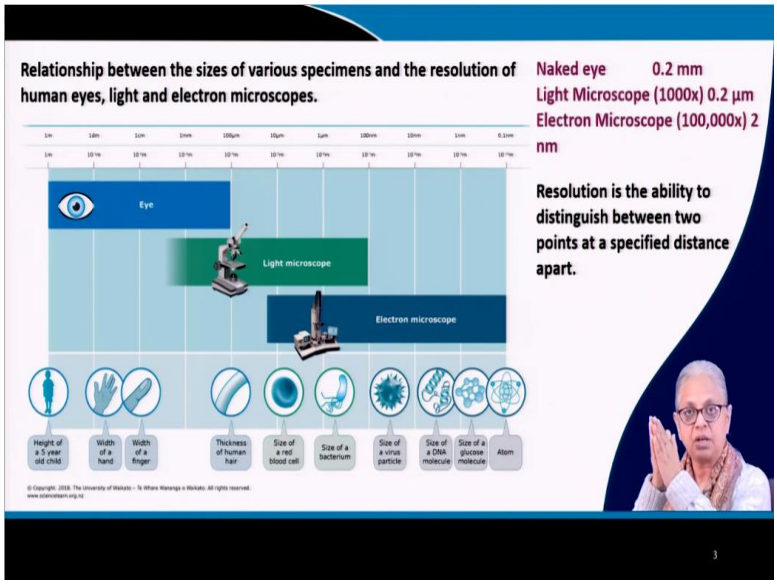
Module - 8
Lecture - 42
Microscopy - I

Welcome everyone to lecture number 42. And this is a new topic that we are starting today. And it is one of the most important topics in Environmental Microbiology. And that is Microscopy. So, we are going to go through two parts. And it is divided into 2 parts. And we are going to go through some of the developments in microbiology; how do we visualise microbial organisms; and what are the limitations of these methods. So, we are going to go through all of that.

(Refer Slide Time: 01:02)



(Refer Slide Time: 01:04)



So, before we get into the details about microscopes and their limitations, let us take a look at the spectrum of different objects that we have around us and what are their sizes. So, when we look at the height of a human being, let us say a child, that is about a metre. A five year old may be about a metre; five to 10 year old may be about a metre tall. The width of your hand is one tenth of a metre. So, that is 1 decimetre. And the width of your finger is approximately 1 centimetre. So, that is what you see over here. And then 1 millimetre, what can you think about? You can think about a grain of sand. So, coarse sand is just about a millimetre thick. And then, we have the thickness of the human hair. That is about 100 micrometers. So, going from 1 metre to 1 millimetre, that is thousandth of a metre. So, 1 millimetre, you know. And going from millimetre to micrometer, that is another thousandth of a millimetre. So, 100 micrometers is about the thickness of a human hair. The size of a red blood cell, you can see it under an optical microscope. You may have gone to any lab or even within your school lab, you can probably do this even in the lab. That is about 10 µm. And then we have the size of a bacterial cell. A bacterial cell is, like I said, on an average, the size of a bacterial cell is 1 micrometer. That is a gross approximation, but it is a reasonable approximation.

So, as I said, after the bacterium, the smallest biological particle would be a virus particle. These viruses can range from anywhere from 15 nanometers. The smallest virus that is currently known, it is called the porcine circo type 1 virus. It has a capsid diameter of 17 nanometers. It can be 15 nanometers under certain conditions. And it can go all the way to

large viruses called the megavirus, which has about 440 nanometer capsid diameter. It was recently isolated in 2010. And so, that is the range of the virus particles.

Then we come to our DNA, the size of a DNA particle. The diameter of a DNA molecule is considered to be about 2 to 2.3 nanometers. The size of a glucose molecule; we have seen so much of glucose in the last topic; and we are going to be doing a lot more with it in the subsequent topics. Glucose is considered to be the simplest and easiest example of an organic compound. Therefore, most of what we talk about in terms of metabolic pathways and everything else that we do, is generally with glucose as a starting compound. What is the size of a glucose molecule? So, along the long axis of the molecule; remember the ring form. I said glucose is found in ring form. So, the length of the glucose molecule is about 8.6 angstrom; and the width of the molecule is 8.4 angstrom. So, it is a ring. And rings are generally considered to be circular and symmetrical. But this is like, you have seen in the previous topic. This is more or less like a; it is a hexose and it has a chair form. So, all these things make it the kind of molecule it is. So, slightly longer than it is broader; just a little difference.

And 1 atom is considered to have; again, this is a gross approximation. It depends on the nature of the element you are looking at. But we normally assume that 1 atom is 1 angstrom. An angstrom is 10 to the power -10 metres. What does all this have to do with microscopy? What is my ability to recognise an object in front of me? So, anything in this range; very easy to find, right. I can see anything up to a centimetre, half a centimetre; even less, I can go down to that level quite easily, even with poor eyesight. But what is the limit of the human eye? The limit of the human eye; most people cannot go beyond a fraction of a millimetre. So, up to 1 millimetre. The individual grains of sand; not a problem for most of us to look at. So, if you look at a large amount of sand, you can separate 1 grain of sand from the rest. And you have a rough idea that, that is 1 millimetre or maybe even smaller than a millimetre. Up to that point, most of us do not struggle.

But I want to visualise objects that are much smaller or invisible; what we say are invisible to the human eye. So, we can go down to maybe 0.2 millimetres. I am saying that the limit of the human eye is 0.2 millimetres. Some of you may be able to go down a little bit further. But let us take that as an average. Now, we want to visualise these microbial organisms. These are single celled organisms. Can we look at them?

We know we cannot look at them without some kind of tool. And the best tools that we have all been familiar with for quite some time, a light microscope. So, I think most of you in your high school would have used a light microscope. And these light microscopes; these are also called optical microscopes. These optical microscopes can give you at best 1000 to 1500 times magnification. You cannot really do much with your normal compound light microscope that we all have seen in school and colleges. So, schools and colleges generally have; it is relatively easy to find. It is not in the lakhs, it is in a few thousands of rupees and so on. So, it is relatively expensive, but not impossible for schools and colleges to have. We do have some other light microscopes as well. I will talk about them later.

But it is safe to say that most of these microscopes have an upper limit in terms of magnification of 1000 to 1500 times. So, that means that you can go from 0.2 millimetres, you can go down to 0.2 micrometers. At this level, you can recognise a bacterial cell. So, a bacterial cell, let us say, has 1 μ size. So, I can look at the sample. I can look at it and I can say, okay, that looks like a bacteria.

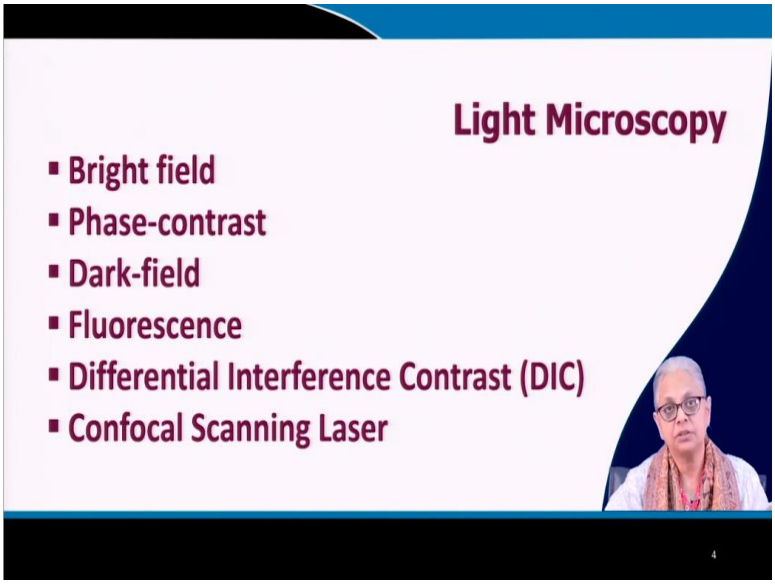
But can I see any details? Can I see the organelles that are there? Can I see flagella? Can I see the DNA? Not possible. We know that even recognising a bacteria; I can tell you from experience that even recognising a bacteria under a light microscope is quite difficult. You can see a protozoa. The protozoa is much bigger and it is easy to see. RBCs which are 10 times larger; they are also easy to see.

But here we are near the detection limit of the light microscope. So, it is kind of difficult to identify bacteria under a light microscope. For that, we have another method and that is electron microscopes. And now, we have several other types of microscopes as well. Now, let us look at electron microscopes. They go down to hundred thousand times magnification, which means you can reach all the way down to 2 nanometers.

Now, there are other microscopes like atomic force microscopy and so on. You can go down to 1 angstrom or maybe even less than that. So, there has been enormous progress in the last, I would say 150 years. There has been a huge amount of progress in terms of increasing the limit of the human eye. So, today, we have the ability to visualise objects all the way down to 1 nanometer or perhaps even less than that; all the way to 1 angstrom and further down.

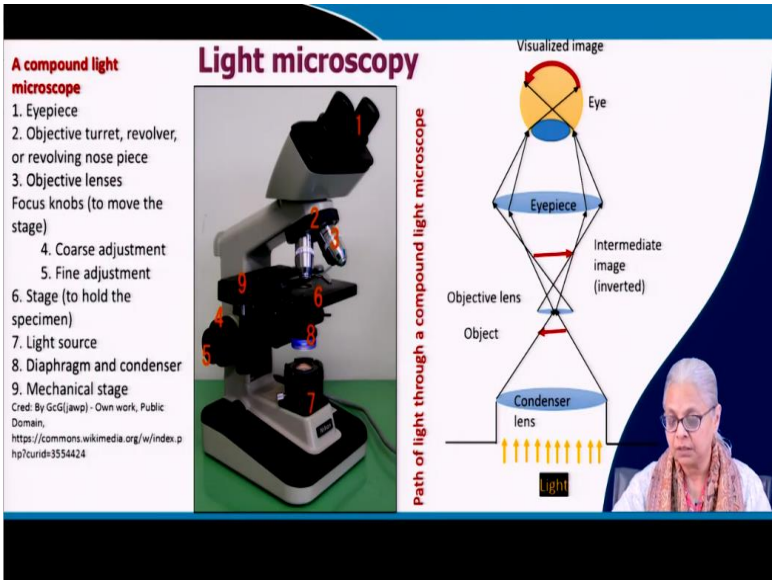
And we will go into that in the second part of this topic. Then we come to another issue related to microscopy. And that is resolution. Resolution is defined as the ability to distinguish 2 points that are some distance apart. Now, if I am looking at some object in the distance; and it is not possible for me sometimes to say whether it is a single object or 2 different objects. When you get closer, you realise that, oh! what I thought was a single object is actually 2 objects. So, resolution is the ability of our eye to distinguish between 2 points. So, if you can see it, I cannot show it to you. But if you can see it; if they are too close, then it is very difficult to see that; no, there is a small distance, right. So that is basically what resolution is all about.

(Refer Slide Time: 10:54)



How do we resolve objects? How are there or rather, what are the limitations when we are trying to resolve objects under the microscope? So, that is what we are going to cover in this particular part. Like I said, this part is light microscopy. And that is what we are going to focus on. So, these are the 6 different types of light microscopes that are now available. And we can get all kinds of results from these different types of microscopes. So, we are going to look at bright field, phase-contrast, dark-field, fluorescence, differential interference contrast and confocal scanning laser microscopes.

(Refer Slide Time: 11:38)



Let us start with the simplest one. And most of you are familiar with it. This is your standard conventional optical microscope. So, it is also called a compound microscope. It is called a light microscope or an optical microscope. You can use any of these terms. This is the microscope. And this is how they all look like. Most of them look like this particular microscope. So, you have your light source at the bottom.

I will start from the bottom up. So, you have number 7 is the light source. And above that you have your diaphragm and condenser. So, that is your condenser lens. So, as the light is coming from the bottom, it passes through the condenser lens. And there is a stage. So, this is number 6, which is the stage. The stage is where you place the sample. So, the sample is your glass slide. Most of the time in light microscopy, we use a glass slide.

We mount our sample on the slide; and whether we dry it or keep it wet. There are 2 options; you can have wet mounts and dry mounts. And then you cover it with a cover slip. And then you come to the objective lens. So, here is the light path; Condenser lens, it focuses on the object which is sitting on the stage. The light passes through the object into the objective lens. So, this objective lens is exaggerated over here.

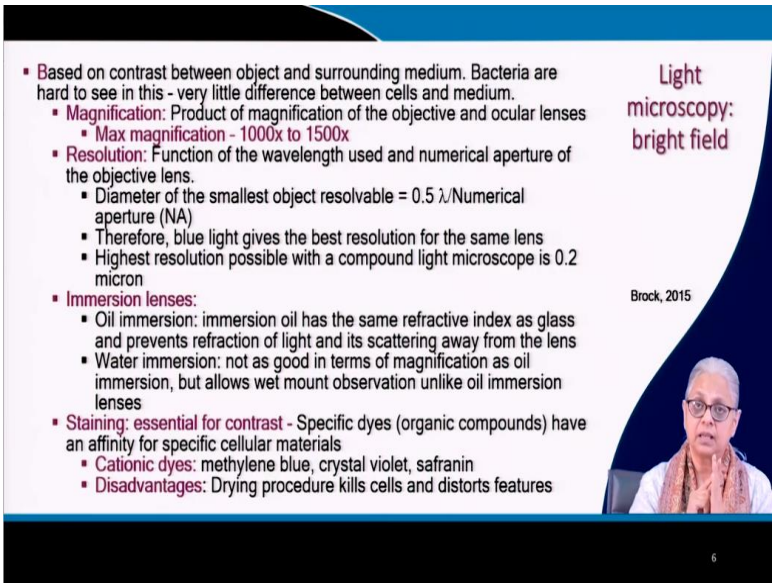
The objective lens is very small. It is like half a centimetre in diameter. And there are; in standard optical microscopes, you will find 3 magnifications, 10, 40 and 100. And right over here, this is the turret or the revolver. So, you can revolve and put the appropriate magnification to focus on the object. And then we have the eyepiece. And the eyepiece is generally a binocular eyepiece to provide stereoscopic vision.

So, if you have a single eyepiece, that will give you no depth information. It will only give you 2-dimensional information. So, stereoscopic information is derived with 2 eyepieces. So, you get binocular vision or stereoscopic vision. The better word is stereoscopic vision. Then we come to the focusing knobs. These are the focusing knobs. The large one is the coarse focusing knob and the small one is the fine focus; so, the fine adjustment and the coarse adjustment.

This is the mechanical stage. And I already mentioned that these days, there has been a sufficient level of sophistication with these light microscopes. So, you have rulers on both sides, in the vertical, in the x and y direction, you might say. So, you can see the calibration or the calipers, whatever you want to call them. You can you can measure the amount of movement in both directions. That level of control is there with the fine adjustment.

And I will add one more point over here. Another major advancement I would say, especially from my time, 30 years ago, more than 30 years ago, when I was working with microscopes. I found that; it was very difficult to count bacteria under the microscope. So, today we have the ability to put in a PC and a camera. So, instead of your eyes, the eye is the camera. So, you have a camera mounted at this point instead of your eyes; and you transfer the images taken by the camera directly to the PC. And it makes storing of images easy; and it makes enumeration of bacteria or any other organisms really easy. So, it is no longer as tedious work as it used to be. And like I said, 20 to 30 years ago, it was awfully tedious work.

(Refer Slide Time: 15:52)



So, let us take a look at some more points about light microscopy, especially bright field microscopy. Now, in our case, like I said, we are going to be focusing more or less on bacteria. But that does not mean other organisms do not exist; they definitely do. Now, so we have an object and its surrounding media. Like I said, unlike algal cells which have pigments in them; and they are much easier to see under a bright field microscope. Bacteria generally do not have pigments. Now, if you have an object which is 70 to 90% water, it has no pigments, it is practically water. So, it is very difficult to see something that is in water and just like water. So, it is very difficult to see bacteria unless you stain them. So, we will come to staining at the end of this slide. That is the first thing. Because there is very little difference between the cells and the media or the water that they are in.

Then we come to the second point and that is magnification. So, what is the magnification of any particular microscope. So, I already said, there are 3 options here: 10 times, 40 times and 100 times. This is the eyepiece. The eyepiece is the ocular lens. And the objective lens is over here. So, the maximum magnification of the objective lens is 100 times. The eyepiece gives you another 10 times magnification. So, the total magnification therefore is 1000.

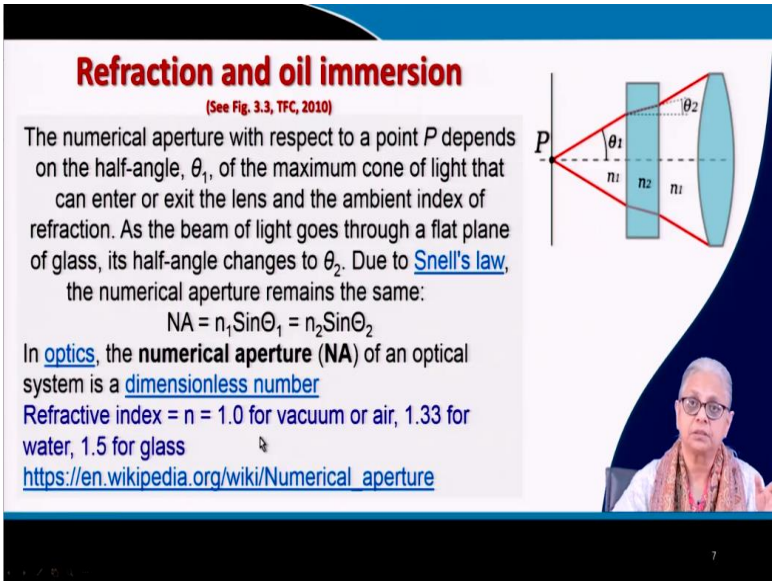
You can increase the magnification by using a particular wavelength. Now, as I said, resolution is the parameter that we use for defining the smallest object that can be resolved by any light microscope. So, the way to determine this particular diameter is to use this formula; 0.5 times lambda; lambda stands for the wavelength of light that you are using; divided by the numerical aperture of the lens. So, numerical aperture is a characteristic of the objective lens. Lambda is the wavelength of light that you are using. Now, in general, in bright field microscopy, we use white light. So, white light has the entire range of wavelengths. But let us say you were going to go for a monochromatic light source. And let us say you bring it down to the smallest wavelength in the visible region. So, the smallest wavelength in the visible region is blue light. So, blue light will automatically give you the smallest diameter resolvable. Resolution is a function of the wavelength and the numerical aperture of the objective lens. This brings us to the highest resolution possible, is 0.2 μm. So, this is the smallest distance that can be distinguished with a light microscope.

So, ideally, you would look at this and say, okay, I can identify bacteria. But from experience, I can tell you, it is too close to the limit of the light microscope to be able to confidently say that this is bacteria. I was working with drinking water. So, in drinking water

systems, the bacteria are starved. They are very small. They are not 1 µ. They are probably smaller than 1 µ. They are probably around half a µ. So, they are almost impossible to look at.

Then we come to a modification within light microscopy. So, you can have what are called wet mounts and dry mounts. I will talk about wet mounts and dry mounts in a little bit. But very often, what we do, especially at the highest magnification of 100 times magnification; we put either oil or water between the object and the objective lens. We do not put air in between. We do not have air in between. Because, one, the objective lens comes very close to the object; and two, to ensure that the light that is passing through the object is collected by the objective lens completely. So, you have a drop of oil and a drop of water, either of them will do. Oil gives better results. This is done to ensure that the light does not scatter away from the object; and it is collected completely by the objective lens.

(Refer Slide Time: 20:49)



So, here is our light source. I have my light source. I have my sample, and that sample is sitting on a glass slide. This is the objective lens. Now, this light source, the rays of light are going to be scattered in a conical fashion. What you will find is that some of the light will pass away from the object; and some of it is incident on the object. Now, our ability to visualise objects, especially in this kind of situation, is that the object has to; if it is opaque, then I can see that there obviously there is some object that is blocking the light.

That gives me one thing. Second is, it has to reflect a colour back into my eyes. So, if it reflects a colour, then I can identify it. So, either it absorbs light or it reflects light; then I can identify an object. If it passes right through, I cannot. And that is what happens with bacteria, because they are practically water. So, it passes right through. If you do not stain the bacteria, you cannot see them. So, that is one issue.

Now, let us take a look at more. So, some of the light will pass away. It cannot be collected, which is poor in terms of microscopy. Whatever comes to the object is going; in this particular case, you have some refraction of light. So, this is θ_1. So, Snell's law says that the numerical aperture of the lens is fixed. So, for the same numerical aperture, you are getting 2 refractions here; one at the first instance; and then again, after it passes through the glass slide. So, at that point, you have θ_2. So, $n_1 \sin\theta_1$ is equal to $n_2 \sin\theta_2$. And n_1 and n_2 are the refractive indices for in the first case for air; and in the second case for glass. Now, if you look at the numbers, n is equal to 1 for vacuum or air and 1.5 for glass. I do not want this change in refractive index, because that is causing more light to be scattered. And what I get in terms of reflection from the object is going to be much less.

So, if I add a drop of oil; oil has the same refractive index as glass; and I will stop some of the light from getting scattered. In fact, I can prevent all of the light, almost all of the light from being scattered. So, oil immersion lenses give us the best results. Now, what about water? 1.33 is the refractive index for water.

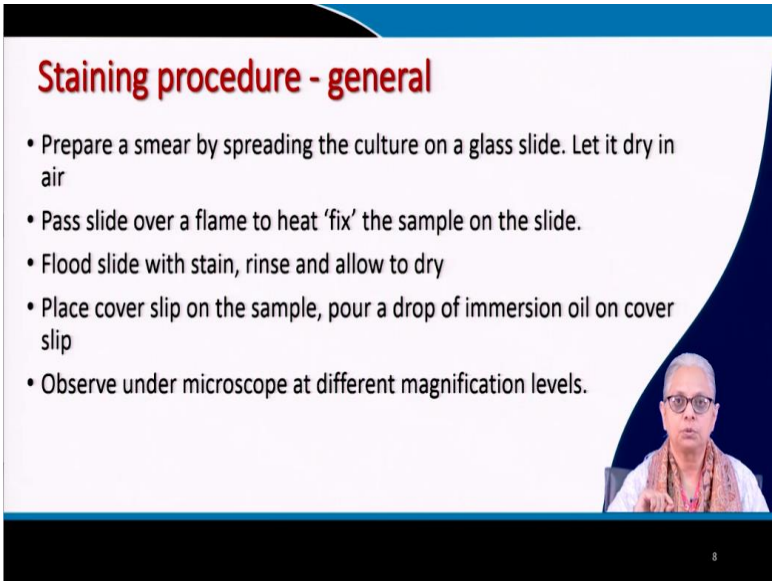
So, if I do not have immersion oil. Now, immersion oil, not emulsion, immersion oil is a special oil. You cannot use any oil that you have around you. No. It is created specifically for this purpose. It is ultra-high purity and it has just the right refractive index. All oils will not have the same refractive index as glass. So, immersion oil is something that is specific for microscopy. That is one issue. And the second thing is, 1.33 is the refractive index for water. So, if you do not have immersion oil, you can use water.

So, water will give you better results than air; less than oil, but better than air. That is about it, about refraction and oil immersion. And there is a lot more in the textbook. There are some very good graphics in the textbooks. So, please refer to them for more details and that will help you to understand what I am talking about. Like I said, oil immersion gives you the best possible results as compared to air and water.

Even though water gives you somewhat better results than air, the biggest advantage is that you can look at wet mounts. So, I can tell you again from experience that, if you take a drop of water and you just want to play around and look at what is there in a pond, let us say a pond or a river water sample. You allow it to; you do not put a cover slip on it; you allow the objective lens to be immersed in the drop of water.

You can literally see it teeming with living organisms at the microbial level. And so, it is a very easy way to look at life without killing it. So, water immersion is a very good way to be introduced to wet microscopy, if you want to call it that. Then we come to staining. Like I said, there are 2 ways of doing things. There is dry mount and wet mount. So, the dry mount is when you dry the sample. And let me show you that.

(Refer Slide Time: 26:09)



So, this is staining procedure for dry mounts. Most of the staining that people do is generally dry mount staining. You can do wet mount staining; but again, it is not very frequent.

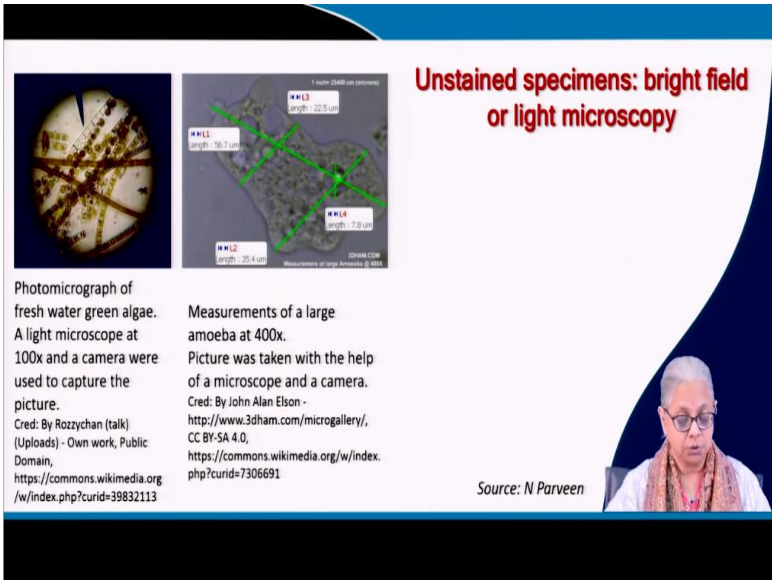
So, let us take a look at the staining procedure. You can refer to the graphics in the textbook. They are very simple and easy. But what is done is, you take your glass slide, your microscopic glass slide; you take your sample; spread it on the glass slide and let it air dry. Let some of the moisture be evaporated. And then, to ensure that there is no moisture left, you pass it over a candle or a Bunsen burner or some other source of flame; and you apply heat to the sample to fix the sample. So, once the moisture has been taken out of the sample, you will get only the solid material that is stuck to the slide. Then you add a stain. Your stain

can be a simple ink. It can be methylene blue. We used to use methylene blue. You can use crystal violet. You can use any stain. And you flood the slide with any stain and then rinse it with water. So, whatever organic material is there in your sample which was already on the slide, it will pick up that dye. And when you rinse it, all the excess will be washed out and then you allow it to dry again. You then place a cover slip that is also made out of glass. It is much finer and smaller in thickness than the slide. You place the cover slip on the sample; put a drop of immersion oil on the cover slip; and you observe it under different magnification levels or the objective lenses.

So, you can have specific dyes for specific cellular materials. Now, different parts of the cell; we have already seen different cell organelles and so on. I have already mentioned the fact that India ink has an affinity for the spore and not for the capsule and so on. So, I have given you all those kinds of examples. So, you know that specific dyes have affinity for specific cellular materials. So, you have cationic dyes like methylene blue, crystal violet, safranin. These are some examples that can be used for staining and observing bacterial cultures. The disadvantages of staining, like I said, they are generally dry mount staining procedures, and the drying procedure kills the cells.

I remember the first time I looked at a microscope. It was a water immersion microscope. So, I took a pond water sample; put it under the objective lens; and I was able to see all the microorganisms in that sample swimming around; the paramecium ingesting the bacteria. It was a living mount, not a dead mount. So, the only disadvantage of staining is that, you are looking at dead cells. The drying procedure kills the cells and it can distort the features of the cell.

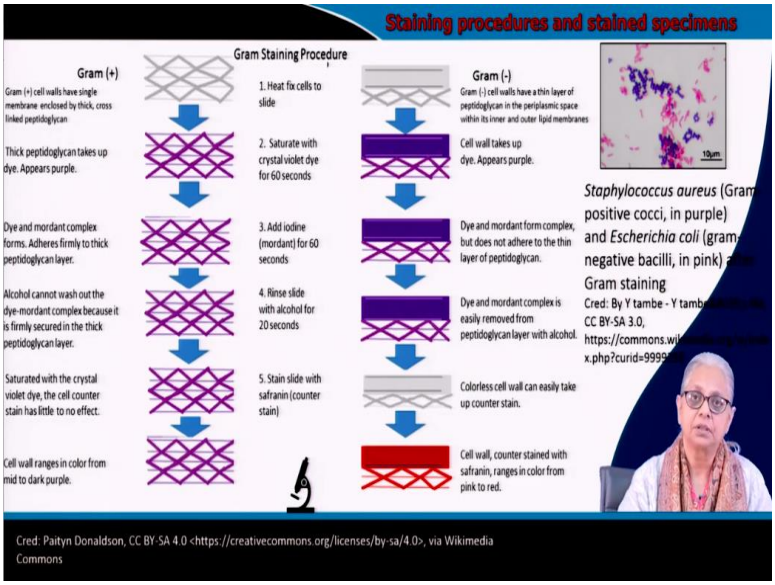
(Refer Slide Time: 29:30)



So, like I mentioned, algal cells do not need to be stained. They carry pigments of their own. They have chlorophyll in them. And because of that, they have different colours. Like I said, red and green are very common. You have blue-green algae; you have green algae; you have yellow coloured ones; and you have red ones. So, you can see one example over here. It looks yellow, but it is actually green. It is a photomicrograph of a freshwater green algae.

So, anytime you go to a pond, you pick up a pond sample. It will have some amount of green algae in it. And you can see it with a light microscope. So, in this particular case, it is a light microscope at 100x magnification, which means actually 1000 times. And a camera was used to capture the images. So, like I said, one of the new things that has happened in the last perhaps 20 years or so, maybe even more; cameras are available as attachments; and it is now easy to capture images. This is a large amoeba. Now, one of the other advancements in microscopy is that with the use of a camera and a PC and software, appropriate software, you can now measure the size of the objects. So, here is an example of measuring the size of the object. It used to be impossible back in, when I was in school and college. So, that you can see is an amoeba. And the length of this cell is about 56.7 µm. And you can see that the width has been measured at different points, because it is an irregularly shaped cell. So, you can see how different points on the cell have different widths. And that is another one. So, you know, with all the technical advancements, with light microscopy, you can do a lot more today than was possible in the last maybe 30- 40 years ago.

(Refer Slide Time: 31:35)



Let us now come to a very famous staining procedure; and that is the basis of classifying bacteria as gram-positive and gram-negative. So, if you remember the 3 domains, we have archaea, bacteria and eukarya. So, out of the bacteria, we have 2 groups gram positive and gram negative.

So, let us take a look at gram staining. Like I said, gram staining is one of the most important techniques that has been used for a very long time. I am giving you all the reasons. So, why did we go through the structure of the cell wall? Why do we look at gram staining? How important is it? Because, this has been the basis of categorizing all the bacteria that you see around you into 2 groups: gram-positive and gram-negative. Let us go through the staining procedure, before we come to why it works the way it does.

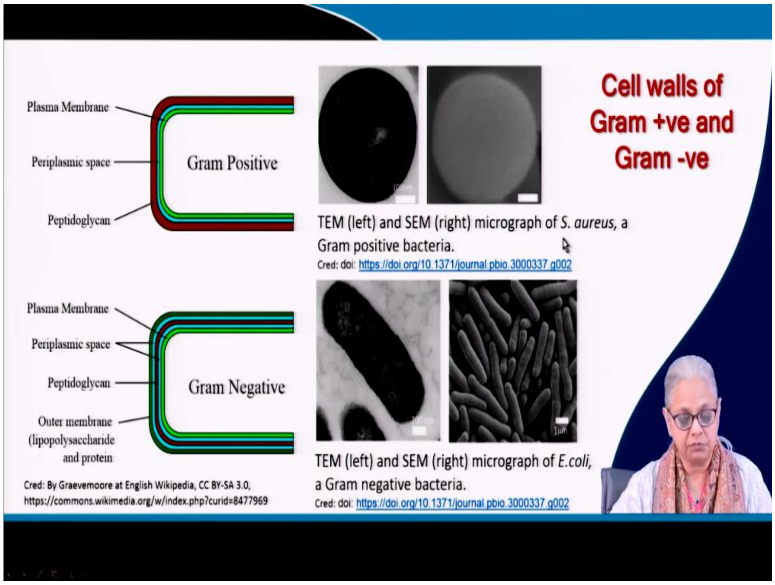
So, here we have the gram staining procedure. There is an example over here of the specimens after they have been stained. So, you can take any sample. You can take a water sample, river water sample, pond water, wastewater, anything. You take the cells; there will be a mixed population of bacteria in any natural water sample. You take the cells and heat fix them to the slides. So, I have already mentioned how heat fixing is done. And then, you apply crystal violet dye for about 60 seconds. When you dye it with crystal violet, you will get purple colour for all the cells. So, all of them will look purple. Then you add iodine. Iodine is a mordant. It forms what is called a crystal violet iodine complex. So, this complex which is insoluble in water will be formed. This complex is known to attach itself to peptidoglycan. So, we have already seen in cell biology that peptidoglycan is present in the cell wall of both

gram-negative and gram-positive bacteria. What is the difference? Gram positive has a thick peptidoglycan layer; and gram negative bacteria have a very thin, maybe a single layer of peptidoglycan, and gram negative bacteria have an outer membrane. So, there are 2 membranes in the gram negative bacteria; an outer membrane, a thin peptidoglycan layer, followed by the inner membrane. Now, all of them, in the first case, will become purple. And this graphic represents the cross-linked peptidoglycan layer. So, all of them will look purple.

Then you rinse the slide with alcohol for 20 seconds. When this is rinsed with alcohol for 20 seconds, you will find that many of the cells have disappeared from view. And those have become colourless. So, the thin layer of peptidoglycan is holding on to some of the purple colour, but the outer membrane has been destroyed by alcohol. So, it is the loss of the outer membrane by alcohol that causes decolourisation of these gram negative cells. The thin layer of peptidoglycan will retain some of the crystal violet iodine complex, but not sufficient to be visible, because it is a very thin layer; it is considered to be a monolayer almost. So, this is practically colourless. It cannot be seen. Then you counter stain it. Just to make it visible, you stain it with a counter stain called safranin. Safranin is orangish, reddish in colour. So, the cell wall as well as the peptidoglycan layer will now show as pink or red.

And the inner membrane is intact. The peptidoglycan is intact. So, you can still visualise the cells. And you can see this over here very clearly. So, you have pink cells and purple cells. The gram positive cells are purple. These are *Staphylococcus aureus*. And the others are rod shaped; and that is *E. coli.* And that is a gram-negative bacilli in pink. So, here you can visualise very clearly the shape as well as the size of these different types of bacteria.

(Refer Slide Time: 36:41)



So, more about that. I have already shown you schematics in the previous topic about gram positive and gram negative bacteria. Here are some more examples of transmission electron micrographs and scanning electron micrographs of *Staphylococcus aureus*, the same gram positive bacteria. And here is *E. coli.* You can see the sizes. It is 1 µ in diameter and about 5µ in length over here.

If you feed it rich nutrient media, if you grow it in rich media, you will get some long ones and so on. So, here is a 200 nm (scale) transmission electron micrograph and these are scanning electron micrographs.

Thank you. That brings me to the end of part 1. We will complete the rest in the next part.