Applied Environmental Microbiology
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Lecture - 03
Cell Structure I

Hello students, welcome to the third lecture of applied environmental microbiology. In the previous two lectures we explored the realm of applied environmental microbiology and looked how the invisible world of microbes influences our health, our environment and our earth. Today we are going to explore in this lecture; how we get information about the microbial world?

We human beings are very visual creatures and as such its very important for us to be able to see what we are studying? And in one of the previous lectures, we had talked about how discovery of microscope was a frontier and groundbreaking invention in the field of microbiology. Because microscope allowed us to look at and observe the microbial communities and phenomena and today we are going to explore the microbe microscopes that we have used and are using currently. And what we see when we look into the microscope and peer down to the slide that has our microbes on it.

So, today this lecture is titled cell structure; one of the most influential microscopic methods today is light microscopy.

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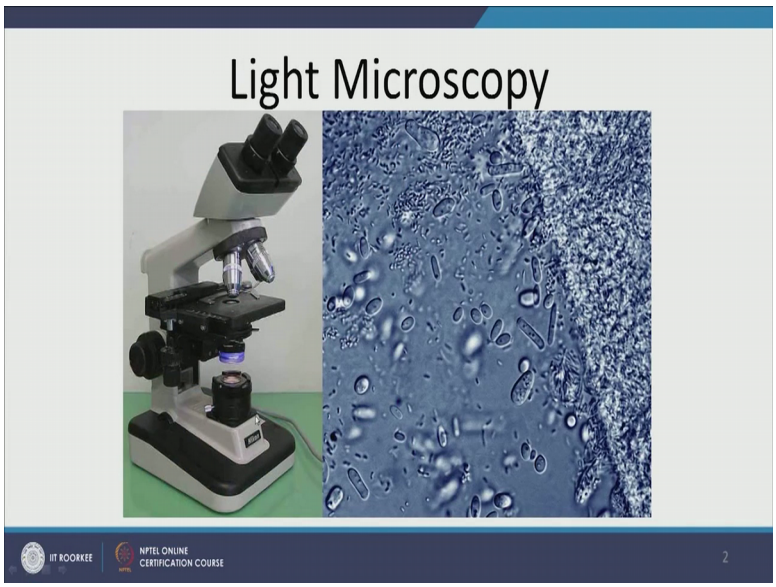

This is something that you are probably familiar with from your high school biology class. This is a microscope that shines light on the sample has a decent magnification where you can definitely see eukaryotic cells in all their glory and prokaryotic cells swimming around.

Simple as its structure is and despite its cominis; in fact, not limited by its common popularity, light microscope is still used in many high end microbial laboratories for the simple reason that they are still very useful for observing cell activity.

Well this picture on the right is a static image seen by light microscope, it has different kinds of cells in it; eukaryotic and some prokaryotic as well. It is important to note that here what you see is a static image of what you might actually observe under light microscope. More of and them; now we see these small microbes undergoing motions, they move around either it is Brownian motion or it is actual microbes whipping their way around in the solution.

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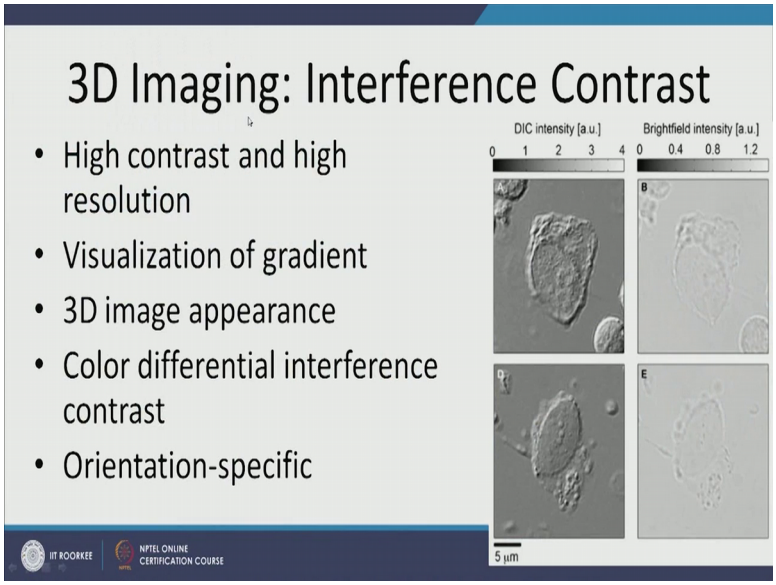

The another form of microscopy that is very popular and high end in this regard is 3D imaging, which actually allows us to get a 3D impression of the microbes; because again human being visual creatures like to observe world in 3 Dimensions and this is interference contrast microscopy, which allows high contrast. So, we can actually see the boundaries of the cell its morphology more clearly with higher resolution in higher contrast.

And because it allows 3 Dimensional imaging, we can see gradients of all types within the cell and on the right. If you observe we have 4 panels the left panels top and bottom are DIC, which is interference contrast microscopy differential inter interference contrast microscopy. And on the right side panels are bright field intensity which is images captured by light microscope that we studied earlier here.

So, we see from light microscope the contrast is not very beautiful we can barely make out the outline of the cell. Same slide, same cell observed under DIC that is differential interference contrast; we noticed the riches and the gradient of the cell where it rises where it ends giving us and it gives us a clearer picture of the cell morphology.

So, note both high resolution and higher contrast and the beautiful 3D appearance that we can observe here. If we use different panels and different filters in our DIC microscope; we can generate color contrast as well. And as you can see because its 3 Dimensional microscopy we can its very it is orientation specific. So, something that we see from one side may appear very different from the other side.

The other kind of microscopy that is very popular nowadays in microbial can research our communities is atomic force microscopy.

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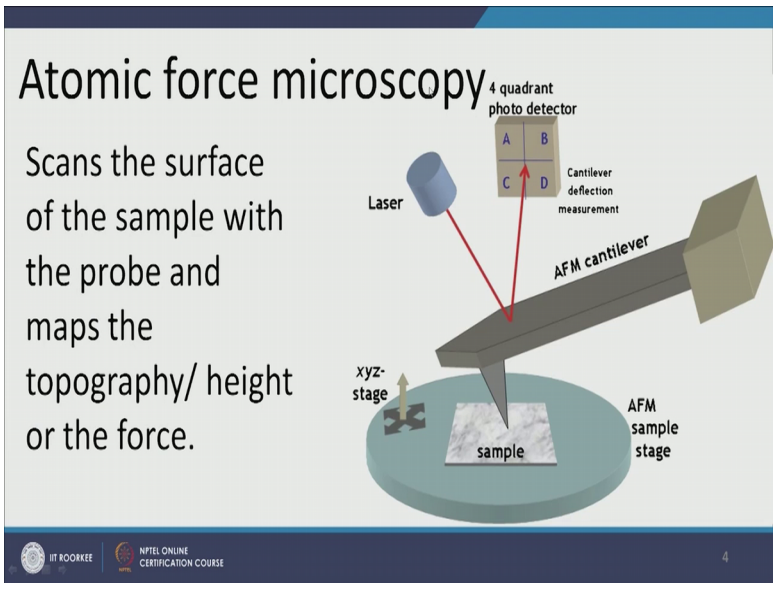

Now, atomic force microscopy is a beautiful instrument whose inventers actually won the well price for this, where we have a cantilever that holds a very small sharp pointed

needle. On its edge; it is nano scale and we have sample laid on a very stable and leveled stage. And this cantilever is moved around the sample and the while its pointed needle interacts with the sample.

Now, ask the sample according to the samples topography; the needle moves up and down and records the height of the sample. Now a very obvious question that might be arising in your mind is; what if sample and this needle are interacting with each other on a chemical or physical scale. Well there is a very common phenomena and it is a very good question by the way.

It is a very common phenomena that we observe in AFM; that sample and the tip of the needle interact with each other and that interaction is also noted and that is on the left I have written; that AFM's needle scans the surface of the sample with the probe this is the probe and maps the topography height or the force, which gives us information about both the physical morphology of the sample, it also informs us about some information about chemistry. AFM is used very frequently in other technology such as nanotechnology and electrical sciences and we are very lucky that we are able to use this in biological sciences as well.

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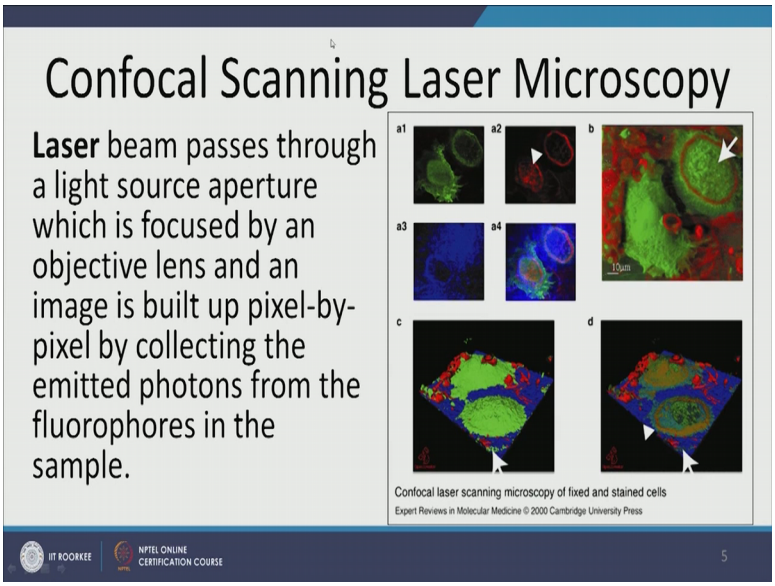

The other kind of microscopy that is very very common and prevalent, we have this in IIT Roorkee as well and most IIT is most research institutes and universities in our country; Confocal scanning laser microscopy. Now images on the right perhaps are the

most beautiful of images that you have seen until now. So, we had bright field microscopy that gave us well this is a really nice picture of bright field microscopy by the way.

But it gives us some idea about how many cells are there? And where they are? And perhaps how they look like? Then we have DIC that gives us 3 Dimensional image and a better understanding of the gradient. And then we have AFM which gives us nearly nano scale morphological features and information about our sample. And now we have Confocal scanning laser microscopy which is very very common in microbial techniques such as fish.

Now, fish is often coupled with Confocal scanning laser microscopy and what fishes I will be talking about it later in few lectures some lectures from now. But for now it is important to know; it is enough to know that what we do when fishes we take our microbes and we add dice to them. And different kinds of dyes attached to different kinds of micro a very rough example here would be.

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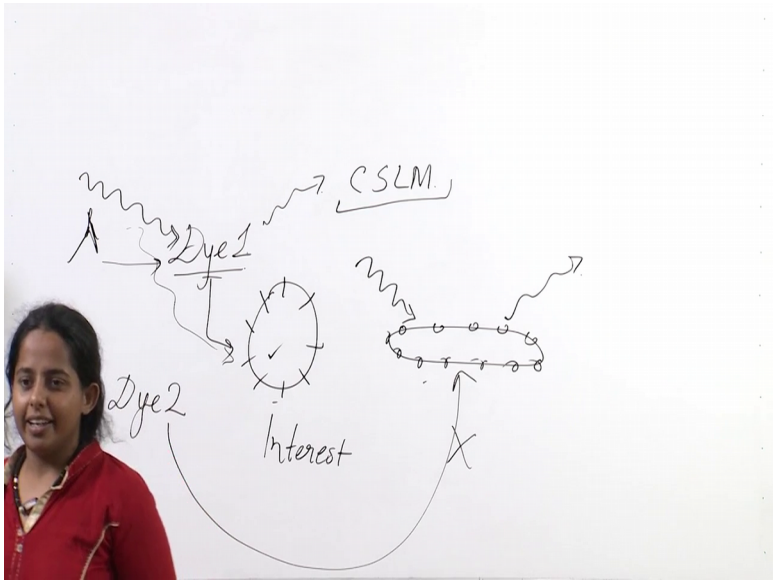

Let us say in a microbial community we have one cell like this and then we have another ROD cell. So, this is a spherical cell somewhat and this is a ROD cell and we have added a dye 1.

Now, the peculiar characteristics of dye 1 should be that it should have the ability to fluorescence under a particular laser. So, what it means is that wherever dye 1 is; if I illuminate laser of a right wavelength, then the dye 1 will give a signal that can be caught by a Confocal scanning laser microscopy. So, Confocal scanning laser microscopy will pick the signal.

Now, when I add dye 1; let us say dye 1 is specific to only the bacteria I am interested in and let us say this is the bacteria of my interest; this is the bacteria I am interested in, not interested in. So, I add dye 1 here and the dye 1 attaches to the cell membrane of this microbe.

Now, when I will shine a particular the right wavelength laser on this microbe, it will give a fluorescence signal which will be picked by Confocal scanning laser microscope. And I can get an idea of the cell microbe morphology and its presence here, I can make it doubly cool if I add another dye; dye 2.

Now, let us say dye 2 is specific to the other microbes present in this microbial community. So, dye 2 will come here and attach to the surface of these bacteria and when the right light is illuminated on this dye; it will give away a signal, a distinct signal that will be picked up by CSLM.

So, when I have both microbes present together in a singular sample and I am viewing them under Confocal scanning laser microscope, I can have multiple filters, but this implies is that I have a laser light and I put a filter that chooser selects for this particular wavelength that fluorescence dye 1; then I will get an image for this bacteria. And when I change the filter; it will illuminate this bacteria and thus I can capture both microbial communities tell them apart and actually even see how they interact with each other. And this is the exact reason, the precise reason why Confocal scanning laser microscopy is very popular.

So, here we have let us read the text because it is very informative; laser beam passes through a light source aperture which is focused on objective lens. Now, if you remember from your high school physics the objective lens covers a very small portion of your sample. And an image is built up pixel by pixel by collecting the emitted photons from the fluorophores in the sample. These fluorophores are your dyes; I am using a very

generic term here because I promise you we will not get into hardcore microbiology, but we will look at how we apply it?

Next we have electron microscopy; this by far will give you the most clear most beautiful images possible.

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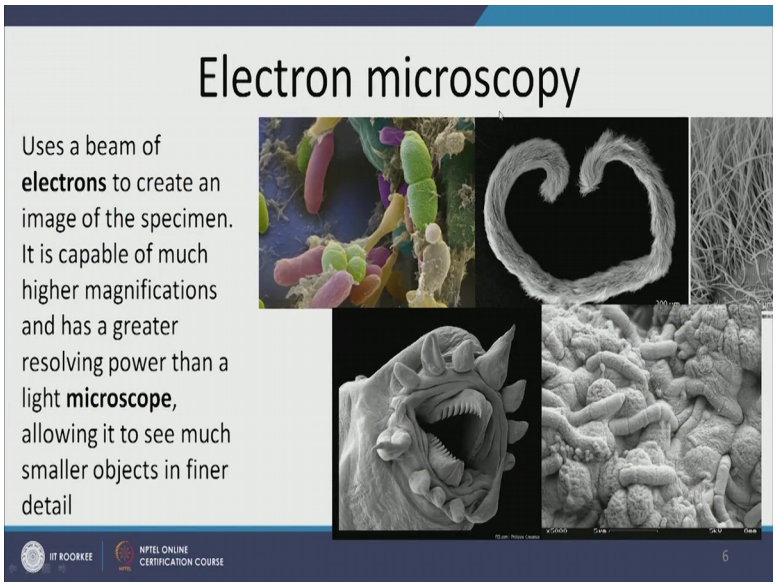

So, let us spend some time looking at and beholding the images on the right side of the slide. Here we have let us look at the top right first these filamentous organisms here and you can note that they are very small; it is not very clear here, but each of these are some nano millimeters in their thickness. So, they are nano sized in their diameter and this is not something we could have definitely; we could not have captured by bright field microscopy; your light microscopy it would have been difficult to captured by DIC.

We would have got a fuzzy image by Confocal scanning laser microscopy, but lo and behold electron microscopy gives us a very very beautiful picture. Then on its left we have such fine detailing of this particular microbe. Now this microbe is some micrometers in diameter and in length and we can get features up to nano scale. And in fact, let me give you some information here, this particular image that we see here almost heart shaped image; the hair like appearance is what has been magnified and seen more closely in the image to its right; so, this filamentous growth that you see are here.

So, not only can we see the structure and the morphology of the microbe very clearly under electromicroscope, but we can zoom in and get a high resolution image. Then on the bottom we have a picture of a biofilm; so this is a microbial community and you can see how beautifully these microbes are have been captured by electron microscopy. On its left we have a wonderful picture for protozoa it almost looks like an aquatic animal, but it is not; it is a protozoa interacting with its surroundings. And above here we have an image again of a microbial community in a biofilm, but it has been rendered colorful artistically.

So, we can take each of these images and color them accordingly to give a false color, but a clearer picture of what is happening in our bio film. What electron microscopy does is, it shines a very powerful beam of electrons to create an image of the specimen. And as its written on the left and is evident on the right, you can gather high magnifications; it has a very high resolution as shown as illustrated by the top rightmost picture.

And it allows us to see much smaller objects in much finer detail and when you get time, I encourage you to look at the right side of this panel, this slide and also explore other pictures electron microscopy pictures online; on Google and see how beautiful images we have now. A visual insight we have into microbial world; thanks to electron microscopy again like atomic force microscopy electron microscope is used in many many other fields as well.

Now, we have these wonderful techniques such as electron microscopy, Confocal scanning laser microscopy, atomic force microscopy and DIC and; obviously, the light microscopy which is found in almost all the high schools.

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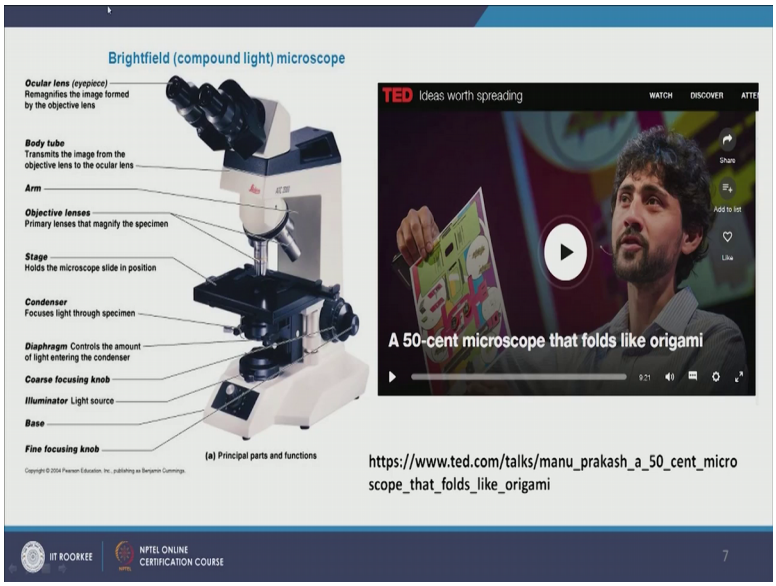

The question is how important and how relevant these microscopes are; in sense that do the directly impact environmental health, public health; can a layman use them because a layman cannot use in an electron microscope.

Let alone use electron microscope, we cannot house electron microscope in a traditional room such as the one I am in right now. It requires special facilities, special care and maintenance and therein comes the beautiful invention; recent invention of Doctor Manu Prakash; who is a British scientist and I encourage you to go and take a look at his ted talk; it is called a 50 cent microscope that folds like an origami.

And he has made a paper microscope with the intention that this paper can be shared and given to anybody; any healthcare worker any environmental worker around the world for very nominal cost 50 cent is like 30 rupees. So, for 30 rupees we can have a microscope we can take us make our sample from water; from soil; from blood and from any other tissue very interested in and take a direct look into it. This is very very helpful for detecting pathogens and in my opinion this is where our science; our environmental microbiology science is headed.

So, we want to couple the high advancements that we make in high end labs, state of art labs with very accessible sensors; accessible technologies. That on one hand; prepare us to understand our environmental microbiology better and give us solutions and on the

other hand help us monitor robustly, rapidly for a fraction of cost. So, here is the video on Doctor Prakash's microscope.

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You know the world is struggling with big problems of challenges with biodiversity climate change, health, hygiene all these big problems.

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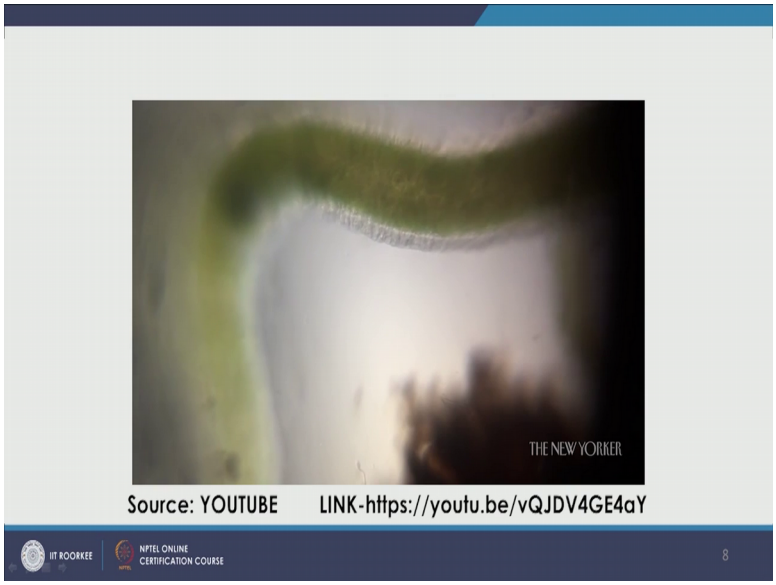

When you start looking at the root of it; actually have microscopic origins.

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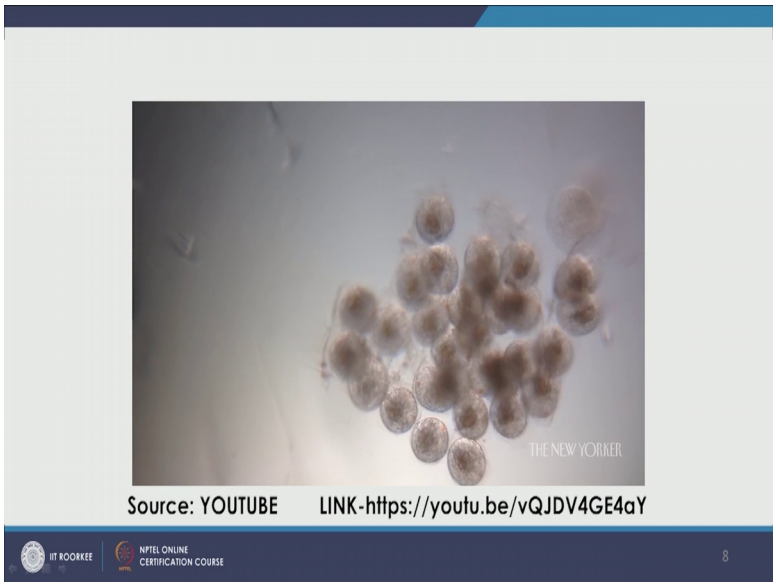

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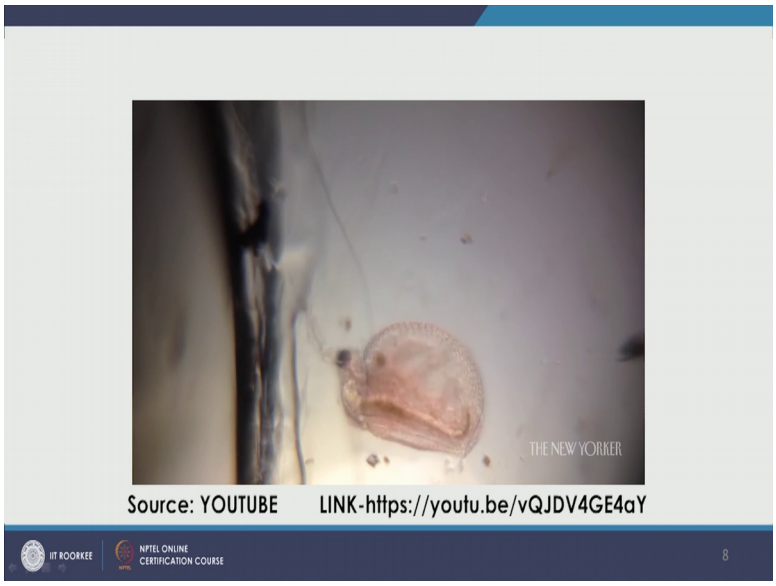

Microscopic things build everything full scope to me is a lens literally into that world it is built out of origami.

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So, it is a flat sheet of paper you fold together and you have a fully functional microscope in a couple of minutes. It weighs less than ten grams and in the end you get an instrument that can give you 700 nanometer resolution imaging at a price point of roughly a dollar. Eco systems are not just based on large species; so, when I walk in the forest I am not just looking at the big trees but I am also looking at the fungus and the insects crawling around.

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There are insects with wings and fully functional machinery that are even smaller than a dot that you write on a piece of paper.

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One time I was waiting for my train actually to come in this very very very tiny bug landed on the rim of my coffee mug, I trapped it in one of my slides and I was absolutely shocked.

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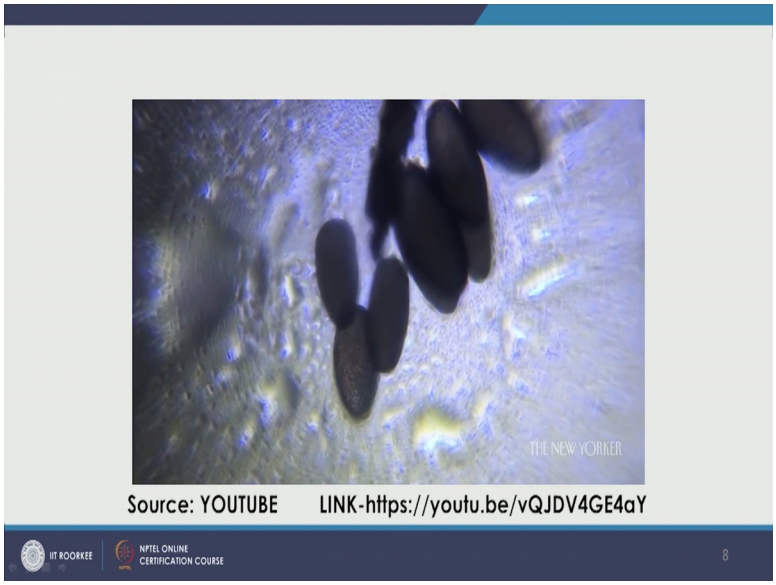

When this insect actually started laying eggs right inside the slide.

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The resources that were dedicated in that insect just for production for progeny is really everything that teaches you something a little about why life is so resilient; it is just such an immense opportunity to come to this world and not know.

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It is not just for scientists to figure out how the world works; that is truly actually a passionate thing that we all start with; we all start by being curious about the world.

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We are born with this and we really need to culture this because fundamentally curiosity needs to be nurtured and kept alive forever.

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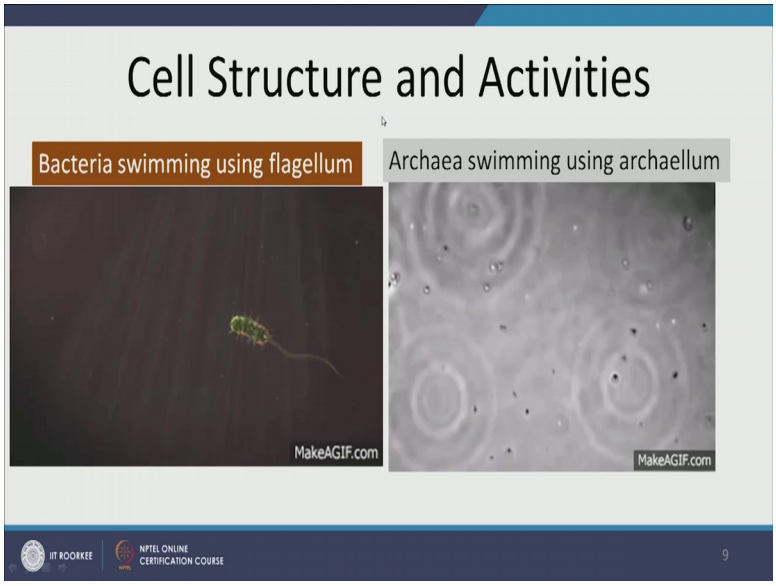

So, using all these microscopes; the one that are high end in using lab and the others that are that hold promise for use in real life, for real life applications; we get insight into microbial communities and their behavior. On the left, we have an image that was captured and then have given false colors of bacteria swimming using its flagellum. And

on the right side we have another picture that captured Achaea swimming using there Archaellum; these are again beautiful videos that are available online.

So, they are making use of all these microscopes we have had a beautiful insight into the microbial world and this image gives you an idea of the scale at which we are at which this subject dwell on.

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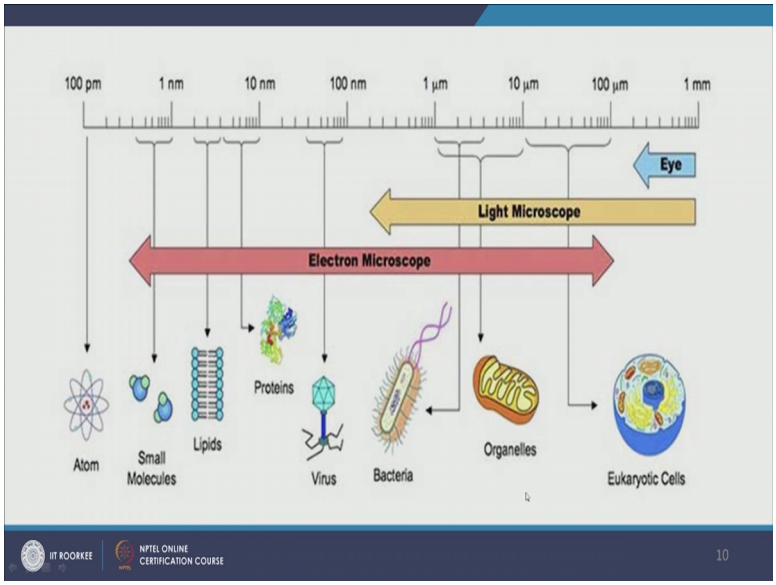

So, a nearly 100 Pico meters and few angstroms we have atoms. In nano scale, we have small molecules, we have fibers, we have lipids or fats and we will talk about them we have proteins and then we have virus. So, nanoscale would include from small molecules; fat molecules, proteins that are very complicated molecules with very heavy molecular weight and virus.

Then above 100 nanometer in non-nano scale; micro scale we have bacteria that is less than 1 micrometer. And then from 1; to pretty good size you know up to 10 micrometers, we have organelles; within these organelles and I will tell you what they are; they are within my eukaryotic cell. And then from 10 to 100 micrometer, we have eukaryotic cell and our eye can; there are only few eukaryotic cells that are visible to our naked eye. So, we can see up to from some fraction of 1 millimeter, but all this is invisible to us.

Light microscope that deals; that is very popularly found in high school, simple labs and even come high end labs can look at things that range from 1 millimeter to little bigger

than 100 nanometer. An electron microscope can capture from less than 1 nanometer up to 100 micrometer. So, you see how electron microscope is very very important and useful to look at the images of lipids.

So, if I am interested in seeing cellular membrane and what their structure is? We want to look at it using electron microscope protein images; we want something like electron microscope bacteria; light field will get a bare idea, I mean we can see them as tiny dots moving around, we can see organelles and eukaryotic cells we can get a good impression. And that is why light microscope is used to popularly for plant tissue and human tissue animal tissue; so, all these are eukaryotic cells.

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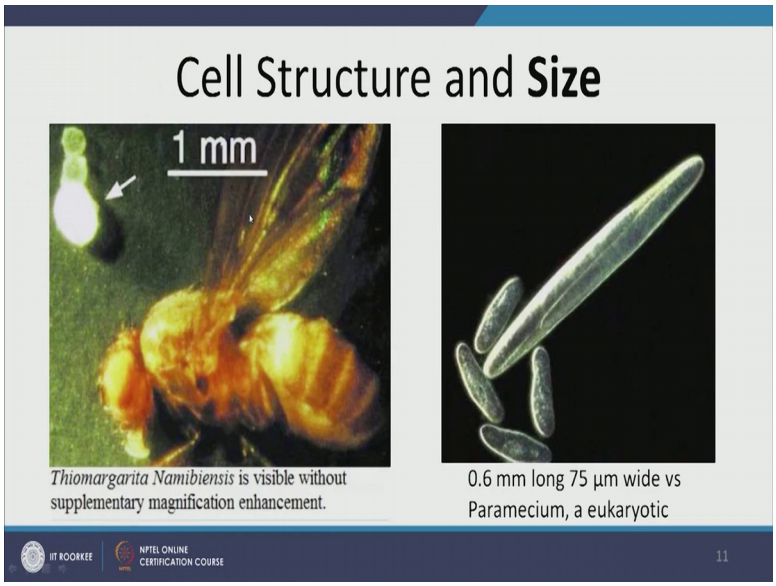

Now, as we were talking about cell structure and cell size and I mentioned to you that very few prokaryotic cells are visible to human eye. This here is your very famous insect that bites and stings and on the left side you have thiomargarita, namibiensis which is a microbe which and is visible without magnification. So, it is one of the large prokaryote that we have and on the right here we have; so, remember here in this we can notice that bacteria which is a prokaryotic cell is much smaller than eukaryotic cell.

So, prokaryote would be around less than or equal to 1 micrometer, a eukaryotic cell would be greater than 100 micrometers. So, eukaryotic cell is 100 times bigger than a bacteria in general, but on right we have a very peculiar image; there are these 4

eukaryotic cells, these are paramecium which are protozoan cells some of them are parasite; pathogens.

And on this long cell here; which is 0.6 millimeter long; we can actually if you really focus, we can see it from bare naked eyes is a prokaryotic cell which is very interesting why would some prokaryotic cells become so, huge that they are up to 0.6 millimeter long? While most of the prokaryotic cells are smaller than or equal to 1 micrometer; and there are many reasons why a cell might; it might be beneficial for cell to get; so, long one is it wants to store certain elements.

So, it gives it storage space; however, from logistic perspective bigger cell is not very advantageous for microbes. So, the bigger is not always more fit, but given its circumstances it might give, it some advantages that a smaller sized cell may not have.

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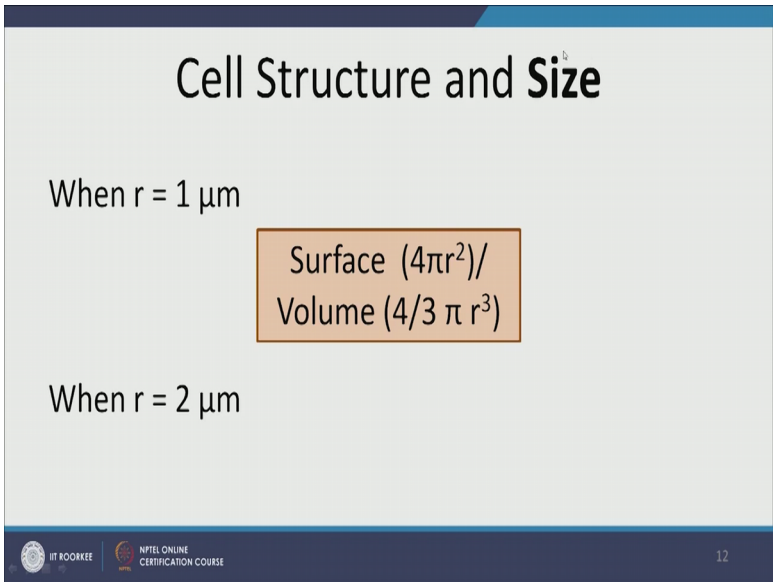

So, now let us try to understand little bit more about size; by size what kind of size is advantageous or not. So, in microbial world we have a very peculiar observation that in general; the smaller the size, the more efficient would be a microbes; a more smaller microbe is the easier it would be for it to gather nutrition and expel the waste.

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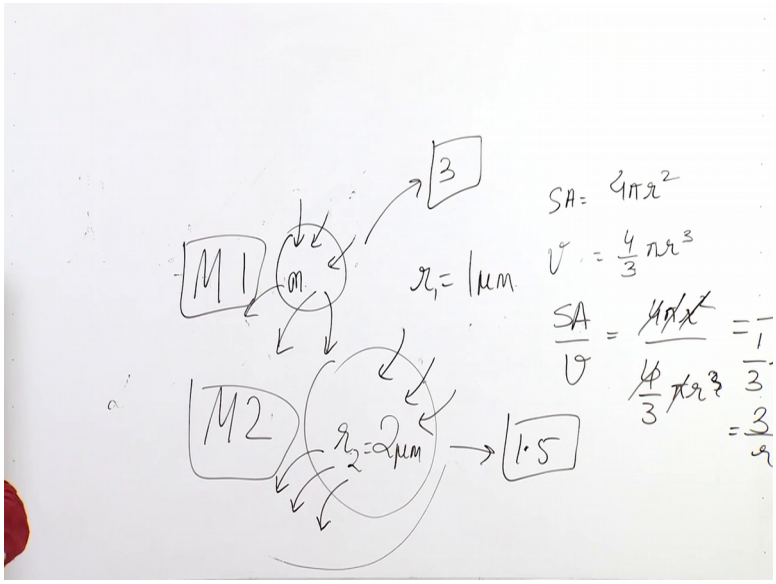

So, let us look here; let us take an example let us say we have two microbes microbe 1 and we have another microbe 2. And these are again false images they are not drawn to scale; let us say the radius of the first microbe is 1 micrometer; 1 micron and radius of second microbe is 2 micrometer.

Now, the question is which of these two microbes would have higher advantage from evolutionary perspective? So, which will be more fit and is likely to survive and how does its size make a difference? Now my dear friends we will find out very soon that for a microbe, it does not live like anybody else; like us to higher orders of life as well. It does not live in isolation it interacts with its environment; so, here is the microbial environment.

So, it interacts with its environment; it gains food the nutrition and the salts that it requires for its sustenance, it sends out wastes, it sends out messages and it sends out other information and even genetic material to its environment. So, it is in a continuous flux with its environment; they are important things needed for survival; that are coming in continuously.

And there are things that are no longer needed for survival or that need to be sent out for communication or other purposes continuously. And same is true for microbial cell 2; if two is living in a flux. So, it is gathering nutrition and food and information from other

cells and environment and it is giving out wastes information and other chemicals such as antibiotic that it requires to survive.

Now, which of these two microbes? Microbe 1 and microbe 2 are likely to survive longer and better. So, for this we have a paradigm; a principle that says the more easily a cell can interact with its environment, the more readily it will have an access to things it needs and the messages it needs to send out.

So, we use a ratio called surface area to volume ratio. So, for any cell that has a higher surface area to volume ratio; for its mass, for its volume, it has higher chances or more surface area, more prospect to share information, share nutrients and enter messages and receive them.

So, let us calculate surface area to volume ratio for both of the microbes. So, surface area to volume let us imagine that they are perfectly spherical. So, for both microbes are surface area would be given by 4 pi r square and their volume would be given by 4 by 3 pi r cube. So, for both microbes regardless of their radius surface area to volume ratio would be 4 pi r square divided by 4 by 3 pi r cube. Now let us make life easier by cancelling the pi on both sides, cancelling the 4 and getting rid of the r square. So, now what we are left with is surface area to volume ratio for both would be or 3 by r.

So, now let us calculate surface area to volume ratio for M 1 and M 2. For M 1 surface area to volume ratio would be 3 by 1 micrometer; so, basically 3. For M 2 surface area to volume ratio would be 3 by 2 or 1.5; so, we can notice here that when it becomes twice the size, its surface area to volume ratio reduces by half. So, here it has surface area to volume ratio 3; here it has surface area to volume ratio 1.5.

So, from logistic perspective microbe 1 with its smaller radius; half the radius is twice more likely to succeed in any environment than microbe 2. However, there is a limitation, a microbe has to have a minimum size; we cannot assume that just because smaller the microbe is the more it will survive; there is a limit to size, it cannot be lesser than the minimum size. And that minimum size is determined by the minimum volume required to store the minimum amount of chemicals and information for life to be independent to some degree.

And now let us talk about structure of the shapes.

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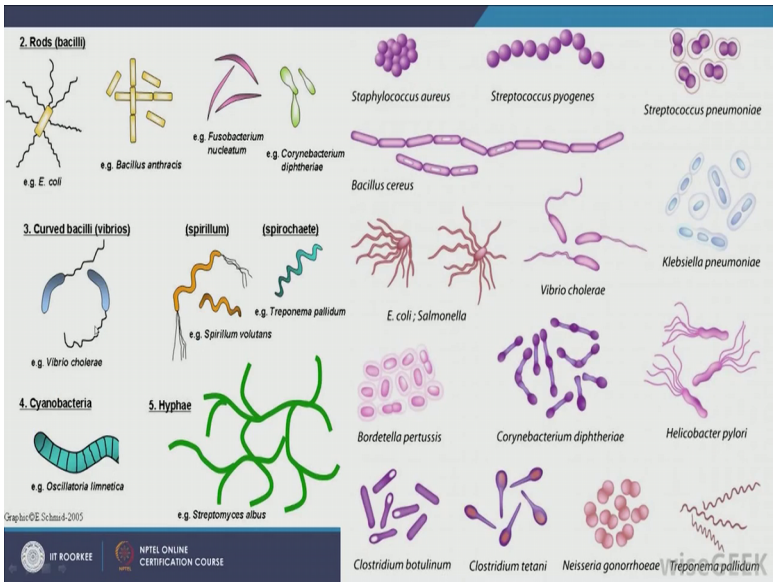

Now, this slide will give you an idea of different kinds of shapes that are we have observed under different kinds of microscopes. Now my dear students; remember that this is only a fraction of what we have observed, these are very general categories. If we take any of them, any single of them let us say staphylococcus oureus which is spherical and lives in closely clustered communities as shown here.

Or if we take e coli; which are salmonella which look very similar or cholera or bordetella, pertussis; each of them will have for example, e coli different kinds of e coli will have different morphologies and each of them have their shape their structure for a very important given reason; depending on their shape their suitable for different kinds of environment.

Let us take few examples, now any of these microbial structures that have these whiplash kind of appendages, these hair kind of structures flagella, pillars. These appendages help them in two ways; one it helps them move in the fluid. And we will in data classes talk about how it helps them in locomotion; the other it also helps them to attach.

Now, structures that are spherical and like microbe 1 and microbe 2; they are just the right surface area to volume ratio for their given environment. Now, it is very important to remember that we will not go into details, but for each of the microbe; the structure that they have evolved with is most suitable for their survival. And regardless of the morphology the essential microbial activities and behavior for example, how do they

grow what kind of chemical bio chemicals are found inside the cell are quite similar across the board.

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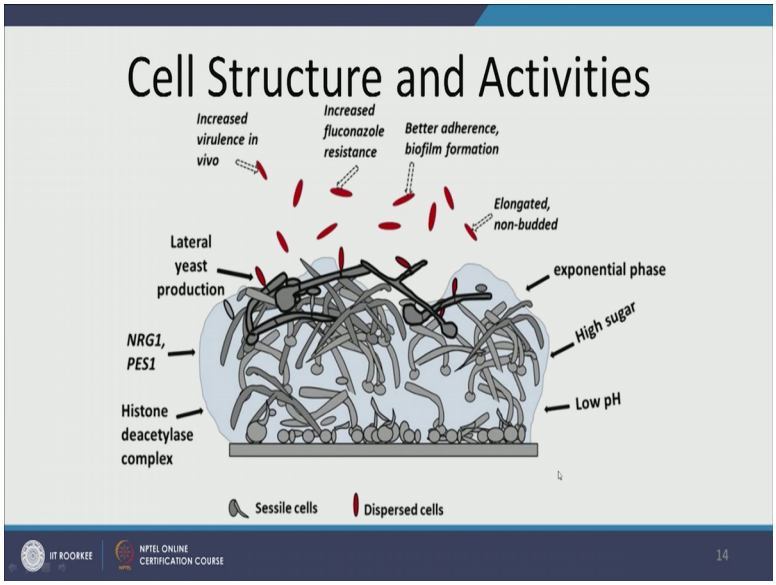

Now, in this image; there is not single microbe, but this is a community of microbe, this is actually a cartoon representation of a biofilm. And there are multiple microbes, there are multiple dead proteins that are not dead protein cells and proteins that are no longer used and in this picture.

But I want to show that even when individual microbes may form different kinds of microbial communities; when it is pure culture; for example, bacillus cereus or we saw in one of the previous lecture; first lecture actually; the green bacteria cyanobacteria. But when they live in complex microbial communities, they can make highly complicated and specialized structures such as biofilms.

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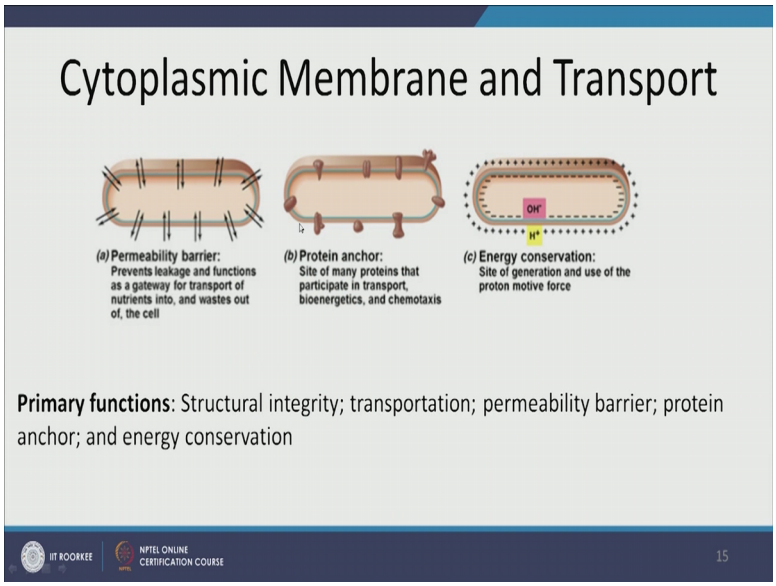

Now, let us dive into what this cell structure looks like; when we talk about cell the first thing that we observe. In fact, for a healthy cell that has not ruptured yet; the only thing we can observe for most microscopy are their outer shell, which often in case of certain prokaryotes is their cytoplasmic membrane. And in cytoplasmic membrane that is the cellular membrane across the cell which is the boundary of the cell more often than not; it has three primary function; one is to give the first primary function is to give it structural integrity.

So, that the cell just not flop away in its environment; does not lose its essential ingredients, so to hold the cell together basically. The other is it acts like a permeability membrane barrier, what it means is that it allows only the chemicals that it needs to come in and only the chemicals that it needs to set get out through its membrane and there is a very nice mechanism that will briefly go over.

And it also acts like a protein anchor and we will talk about it very soon. So, there are proteins that are anchored on the cellular membrane and this is a very beautiful picture here showing it. Some proteins are passed from outer portion of cytoplasmic membrane to inside portion, some of them are only stuck either on the inside or outside.

For example, this one was only exposed to the outside of the cell not inside of the cell. These proteins serve many many different functions for example, transporting chemicals in and out it acting like sensors from what is happening outside, communicating, sharing

genes or not sharing genes bioenergetics that is taking electron from certain electron accept electron donors giving electrons to electron acceptors. So, there is a lot of work that these proteins do very important essential proteins.

The third important function of cell cytoplasmic membrane or cellular membrane is conservation of energy. So, the outer layer of cell membrane is positively charged that is protons and the inner layer is OH minus. And this is essential, this is a very innate quality to a cellular membrane and we will talk about it briefly how. And what this allows; it allows it gives it a proton motive force; a PMF now this acts like a battery for cell whenever cell needs alaine energy to convert ATP into ATP or to send something out or bring something in it can utilize this battery this charge.

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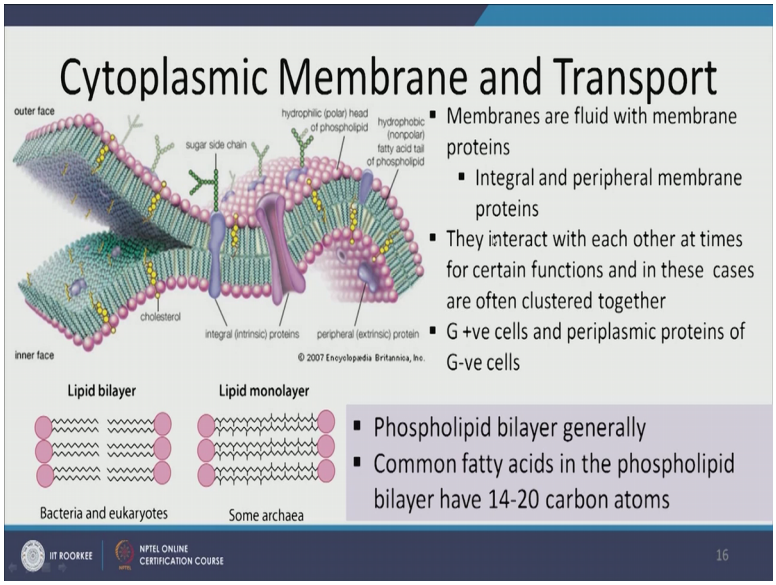

So, this is another picture of cytoplasmic membrane and we will go very briefly over it. So, here you can see in this picture there is an outer phase there is inner phase. So, the upper side is the outer side the lower side is the inner side of the cytoplasmic membrane and this blue color thing is an intrinsic protein, it is integral to the membrane. So, is this purple one and notice that they are through and through; so, these are integral protein that connects from outer side of the set cytoplasmic membrane, to the inner side. And then there are these beautiful bio chemicals and we will talk about them in detail in the next lecture and they have sugars and other bio chemicals attached to them and they all have different functions.

Now, important thing to note is not all the proteins at that are inherently attached to cytoplasmic membrane, not all of them are integral or intrinsic proteins. Some of them are just peripheral proteins; so, they are they are exposed to only one side either outer side or inner side another example is here.

Now, if you notice these lines here represent the lipids; this is hydro phobic portion of cell and the purple balls here are hydrophilic portions of the cell. So, cell membrane essentially on the outside lives in aquatic environment and on the inside has cytoplasm which is basically an aqueous mixture. So, on both sides it has water and how do you make a membrane in water? You make a missile of oil.

So, cell is basically a very tightly held missile of oil. So, the hydrophobic portions stick together to each other whereas, hydrophilic portion are exposed to the outside and the inside of the cell. And these coming back to the proteins; these proteins interact with each other they if they have a lot of functions that they need to perform together they often clustered together.

Now, in this is an example of G plus or gram positive cell; in gram negative cell there is an additional layer here above towards the outside which is made of periplasmic proteins. Now, very briefly our cytoplasmic membrane is in bacteria it is lipid bilayer.

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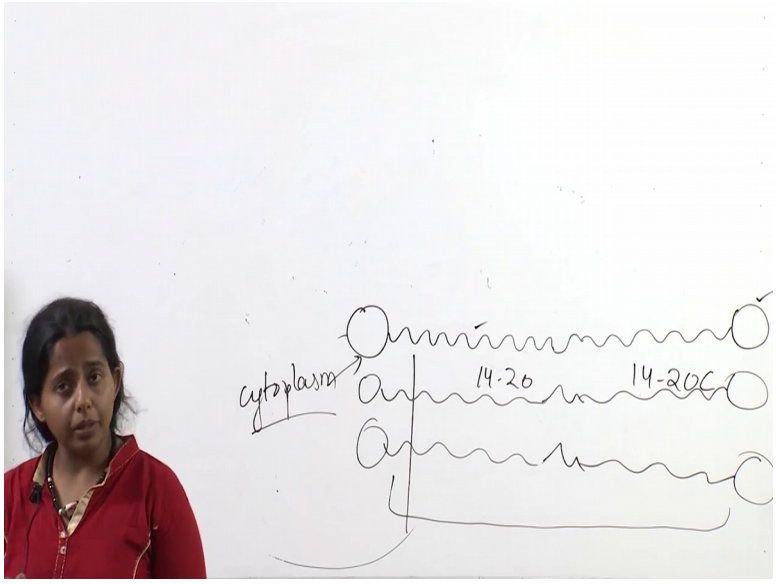

So, what lipid bilayer implies is that; it will have one hydrophilic water loving portion of its membrane and it will have a long fatty chain usually; 14 to 20 carbon atoms long. So, this long fatty chain is hydrophobic because it does not like water it is fatty and this plex.

Now, another similar chemical biochemical with a long fatty chain and a water loving portion will assemble near it in this way. So, we have one layer in this direction; like this and we have another layer facing from here and this is called lipid bilayer because most of it is fatty acid; lipid. So, this is there are two layers one layer and the other layer.

Let us assume that this is the cytoplasm; so, this is the inner portion of the cell and this is the external portion of the cell. Now remember or the environment; now remember cytoplasm is mostly aqueous and the x in cells often need water to survive. So, they live in aqueous environment and this is also water. So, water loving cell assemble outside what living portion of cytoplasmic membrane and water hating stick together.

Now, in Archaea it is very interesting that Archaea do not have bilayer, they have mono layer. What it implies is that this is; how it looks in Archaea, there is one long very long fatty chain and on both ends it has water loving molecules.

So, it does not require another layer to come in complement itself; it is just one continuous layer of molecules that have arranged together to make Archaeal membrane. This is very important; now think about it how it will help Archaea? This would give certain rigidity and certain robustness to the cytoplasmic membrane and it is not surprising that Archaeal membranes are often resistant to high temperatures and can survive in hypothermic environments; really hot environment.

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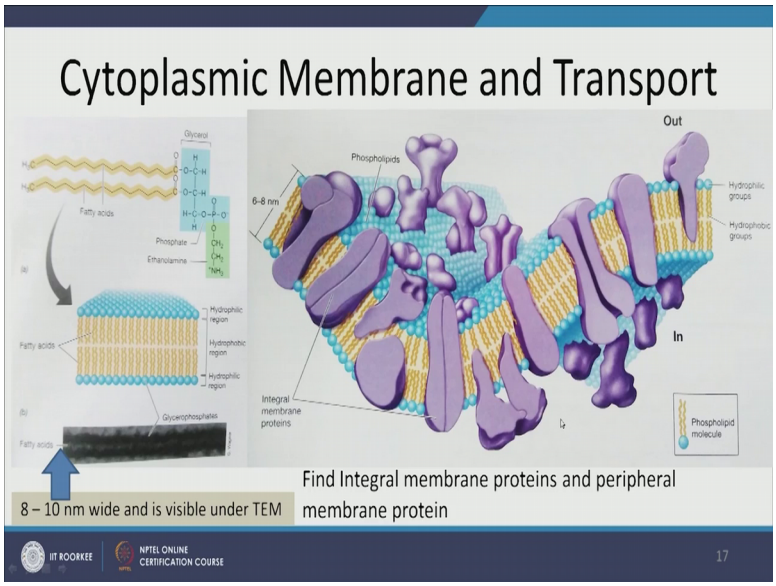

And this is where this is the last slide for today and then from here we will take on to the next lecture. This is another very beautiful picture of cell membrane and I encourage you to take a good look at it, we have already talked about this.

And on the left side; we have what is very beautiful as a TEM image of the cytoplasmic membrane. You can actually see the one black line and another black line both of them are separated by a very faint grey line. So, the faint grey line is what is fatty acids and the black line is glycerophospholipids and we will be parse here for now.

And in the next lecture, we will talk more about the internal structure of cell and the informational non informational molecules. And we will close down the basic microbiology that we need to know for this course and then proceed ahead; so, that is all for today.

Thank you very much.