Techniques of Materials Characterization
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Lecture – 10
Fluorescence Microscopy

Welcome everyone to this NPTEL online certification course. We are right now in module 2 that is we are in the week 2 and we are discussing about various modes of optical microscopy. So, we have discussed bright-field mode. First, we have discussed about reflected and transmitted light microscopy, we have discussed about bright-field microscopy.

Then we went on to discussing about the contrast enhancement techniques, the necessity of contrast enhancement techniques. And then we discussed about another variation of bright-field technique that is dark field where we just change the angle of illumination. And then we moved on to some other kind of contrast enhancement techniques like face field microscopy, polarized light microscopy, interference contrast microscopy and so on.

And today we will be discussing about the last of this contrast enhancement techniques, which is really a special technique that is why I purposefully kept it in the end. Because it is not exactly a contrast enhancement technique like the way all other techniques we discussed phase contrast, polarized light or interference contrast it is not exactly of that sort. It is a technique within itself and it is a very important technique for biologists and life science people.

So that is why I kept it at the end and we will be discussing about this, this is called fluorescence microscopy. And along with that, towards the end I will give you a little glimpse of some few other techniques on optical microscopy which are very specialized techniques, just a very little introduction to them.

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Today, we will be discussing about fluorescence and phosphorescence. These are the two mechanisms or these are the two phenomenon that are the heart of the fluorescence microscopy. And then we will discuss about the components of fluorescence microscopy, principle, how the image is formed in fluorescence microscopy.

And fluorescence microscopy requires a very special type of specimen preparation, so that also we will discuss. And finally, we will discuss about super resolution microscopy as I was telling and one technique we will be discussing here called STED.

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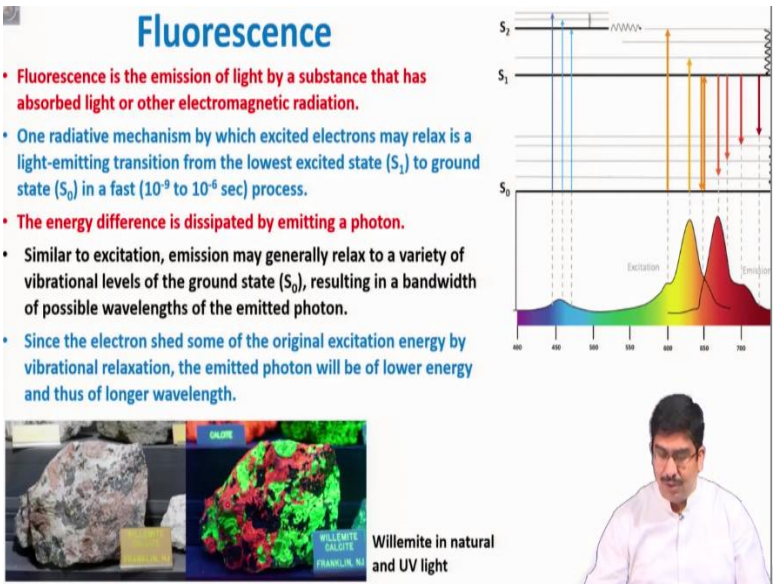

So, first of all let us understand what is fluorescence because as I said it is a mechanism. So, before even going into microscopy, fluorescence microscopy, you need to understand what is fluorescence is all about. So, as this definition goes the fluorescence is the

emission of light by any substance that has absorbed light or any other electromagnetic radiation.

So, basically the way fluorescence happens is true for any kind of spectroscopy techniques. If you take for example the characteristic X-ray which we will be studying within a few more classes. So, characteristic X-ray or any other spectroscopy that we have seen Raman spectroscopy, IR spectroscopy and so on and so forth, so any other spectroscopy techniques what happens the same phenomena happens in fluorescence.

But here the light, signal that emits from the specimen from the material is a visible light is within the visible light that is the difference only. So, what basically happens in fluorescence microscopy as I said like any other spectroscopy techniques or characteristic X-ray generation and so on, so you have a material in ground state and that material somehow gets excited.

The material or atoms electronic transition whatever you can imagine, the electronic transition maybe, so the material from the ground state or the atoms from the ground state they go to the higher energy state and this happens by an incoming signal. That signal can be visible light or that signal can be any other electromagnetic radiation in the range, within this range if it comes and this will excite the material okay.

Afterwards, what will happen is that that material will be now again will try to return to the ground state because that is what the most stable state. In the process, what will happen the material will in this like going into the higher energy state and then coming back to the ground state, in this entire process what will happen is that certain amount of energy will be released definitely from the material because it has gone to excited state, now it is coming back.

So, whatever excess energy is there that energy it will release, that energy, that difference of energy whatever energy is released in this process that will come as a photon, light photon. So, that means ultimately, we will be getting another electromagnetic radiation from this process. So, the first process which is from ground state to higher energy state is called the excitation.

And the reverse process when those from higher energy excited state to ground state this reversal process that process and then a photon will emit that is called emission process. So, this is all about fluorescence. This is actually about a broader process called luminescence and one part of the luminescence is fluorescence. We will see that. So, in fluorescence one more thing happens in this luminescence process that is also very important.

That once this excited, from ground state to excited state, this transition happens afterwards there is a relaxation. There is a vibrational relaxation that happens. These are all quantum mechanical terms we do not need to go into that, just we need to believe that okay yes there is some intermediate level where these electrons or where this material comes back after jump from S_0 to S_2.

And then there is a little relaxation vibrational relaxation and this material comes to an intermediate state, and from there the material finally comes back to this ground state again. So, the photon is emitted based on this much of energy, whatever the energy difference here corresponding to that that photon will be an energy or will be emitted. So, in the process basically we can imagine that there is some amount of energy lost.

Not exactly lost, but the photon is not carrying that much of energy. So, the photon will have lower energy than the original. The photon which is emitted will have lower energy than the original energy that was carried by the incoming signal or electromagnetic radiation. It will have lower energy than that and correspondingly it will have a longer wavelength.

So, the excitation wavelength, if you see it here the excitation wavelength and excitation wavelength will be a large lot, it is a wide range of wavelength possible and this emission wavelength will be longer than that. That means they will carry lesser amount of energy. So, that is what will happen in a fluorescence process and this happens naturally. To many materials this happen naturally. For example, one thing I have shown this called Willemite, it is a mineral.

When you see it under the normal light, you do not see any color anything. When you see it under UV light, now this UV light will work like the excitation voltage and in the

process it will emit some kind of color depending on exactly what kind of a wavelength you are using for excitation signal and depending on that you will be getting exactly a corresponding emission signal and that will come like visible light. So, this is the entire fluorescence sense or a broader sense the entire luminescence process.

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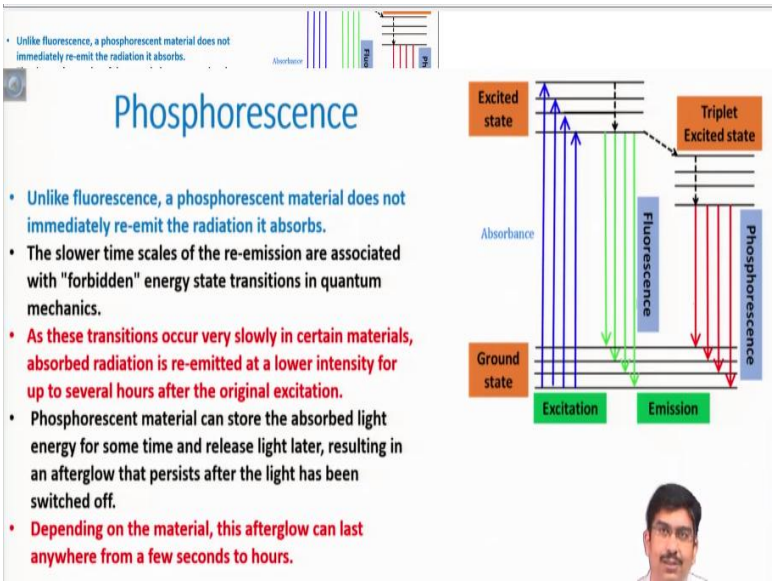

So, luminescence basically actually luminescence, then there is two different luminescence, under that there are two different variations. One is photoluminescence, one is chemiluminescence. Under photoluminescence again the fluorescence and another process that is this phosphorescence. These are the broadly the entire phenomena. Luminescence, photoluminescence, chemiluminescence.

Photoluminescence, again two parts; fluorescence, phosphorescence okay. Now, the phosphorescent material, phosphorescence material pretty much the same thing happens. So, there is an excitation from the ground state to the excited state. Then there is a vibrational relaxation and it comes back fluorescence, this is what fluorescence is all about. Phosphorescence what happens is that from this excited state to the intermediate state.

It will again come down to certain other forbidden energy state, some other kind of intermediate states and this will be a slower process. So, there will be a continuous jump from or continuous relaxation. Here from this excited state there will be a vibrational relaxation, intermediate state, immediately comes back to the lower state. In this case,

there will be a transition between this intermediate state to another some further more intermediate states which are forbidden energy state transitions in quantum,

It is a quantum mechanical again terms, but you can think that this transition is very gradual in case of a phosphorescence. And since this is a phosphorescence, in case of phosphorescence this is a very gradual process. So, there is a continuous emission of photon that will happen. In this case fluorescence case, it is immediately all the photons will come out from here to here and they will almost have the similar kind of wavelength.

Although of course, there will be a range definitely because depending on this intermediate states here, so there will be some other states in their intermediate state. Depending on that there will be some range of wavelengths possible in fluorescence that also is there. But in phosphorescence this range will be very large A and B this will not be a spontaneous process.

So, this will happen even after the incoming initial signal is stopped, the excitation signal even if it is stopped the flow of phosphorescence process will continue to happen, go on and go on because light will be coming out of it continuously and this process is called afterglow. So, this Willemite the moment you switch up this UV light it will again come back to natural state, the light will disappear.

But in case of a phosphorescence material even if you switch off the UV light let us say it will still glow, it will still give the light, so that is called the phosphorescence that is called the afterglow and that is what is a phosphorescence. So, phosphorescence materials give an afterglow even after when the excitation signal is stopped, it will keep on giving the light whereas fluorescence material does not do that.

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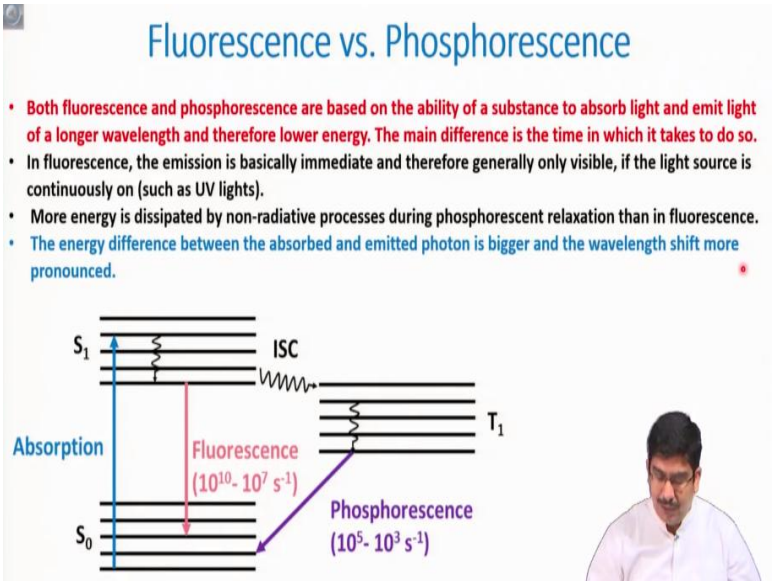

And this you can imagine in a more sort of in a more elaborate way if you look at the time scale at which these entire processes are happening, right. So, as I said both fluorescence and phosphorescence are a part of photoluminescence what is even broader term is luminescence, both of them. But the point is both of them they absorb light, mostly light or UV light, other electromagnetic field either in the visible range or in IR or UV range.

So, they absorb light and then the emit lights, emission usually in the visible light range only. The main difference of course as I said is the time that is there in this process. In fluorescence, it is a spontaneous process, it is an immediate process, and it happens at a timescale which appears to us is an immediate, of course there is a time lag, but this it happens in such a timescale which is pretty much we will not be able to understand.

It is beyond our resolution, the resolution of our senses or of our eyes. So, we will see immediate process, immediately this will happen in fluorescence. But in phosphorescence what will happen is that this process is much slower because now you have some other, it is a continuous gradual transition to intermediate state. So, phosphorescence the timescale will be something that we can understand.

We can see it physically and depending on the material, depending on the light, this can go sometimes from minutes to hours, this afterglow process. So, phosphorescence materials have this tendency. So, that is what the main difference between phosphorescence and fluorescence. Of course, some associated differences are also there.

For example, there is more energy that is dissipated by during phosphorescent relaxation process than in fluorescence that is one thing first thing.

So more energy, ultimately the entire amount of energy almost is recovered in case of phosphorescence material. And second thing in phosphorescence generally the energy difference between the absorbed and emitted photon that is the excitation and emission, the energy difference between these two is more and the wavelength shift that is this process, the wavelength shift here.

This emission and excitation, excitation signal minus emission signal this spread will be much longer or much bigger in case of a phosphorescence than in case of a fluorescence because of this entire difference in the process. So that is it. That is what fluorescence and phosphorescent is all about. And that much if we know we can understand fluorescence microscopy very well.

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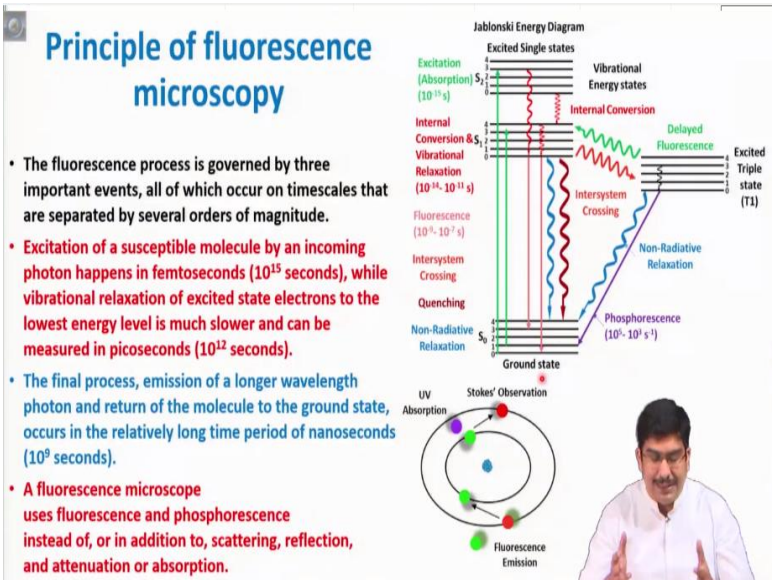

Now, how the fluorescence microscopy basically works. The first thing it takes advantage of this fluorescence effect that is it. So, in the fluorescence effect as I said there are three different phenomena or three different steps or there, three different events are there which has completely different timescales altogether. So, the first one is of course the excitation of this molecule, something, some material.

Excitation of the molecule by an incoming photon. So incoming radiation, electromagnetic radiation is coming and you have a material which is prone to

phosphorescence, usually, it is biological materials. Usually they are called fluorophores, we will come to that. So, you have a molecule which has this ability to show fluorescence. Now the excitation photon that comes and immediately this material goes in the excited state.

So, this process is in the femtosecond range. You can imagine how fast it is, 10^{-15} s. So, it is in the femtosecond range it happens. The next process is the vibrational relaxation as I said. From this S_2 excited single state S_2 to this S_1, this is a vibrational relaxation process. This happens at a much slower speed, this is in the picosecond range around 10^{12} s.

And the final state of course final processes when this one comes down from this S_1 state this comes back to the ground state which produce this photon, excitation photon, excited photon, the signal, final color. This process is relatively longer in the nanosecond range 10^{9} s. But all together this entire process the even in the nanosecond range the process looks so fast that we will not be able to understand.

It will be still faster than what our resolution of our eyes or what the frame per second or whatever the time gap we can differentiate between two events, it will be still faster than that. So we will not be, for our eyes it will be completely spontaneous. So, this fluorescence microscope basically uses this entire process, this fluorescence process, sometimes phosphorescence process as well.

So, fluorescence and phosphorescence both the process it takes advantage of this in addition to the normal scattering, reflection, absorption, all these processes which are there in the bright-field microscopy. So, it is basically kind of a bright-field microscopy on top of that the fluorescence or phosphorescence effect is superimposed. So, we will see how it is done.

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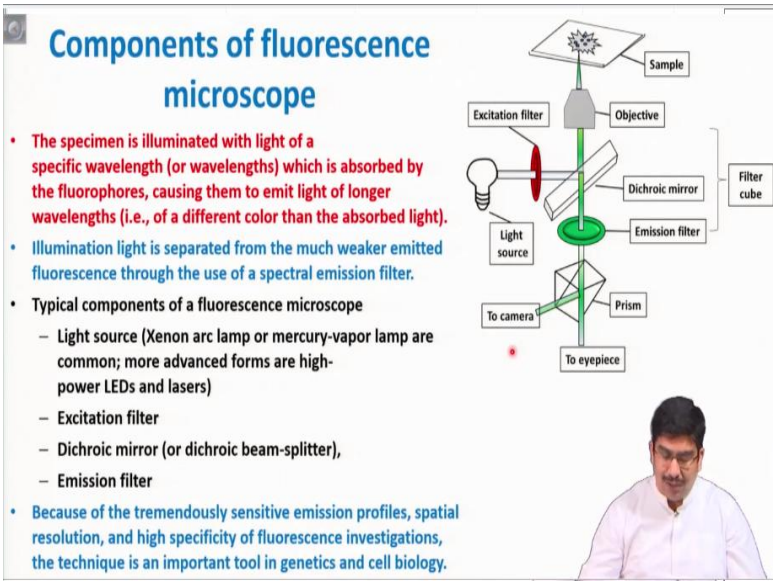

So, the components of a fluorescence microscope as I said here again, you have to generate the fluorescence effect. So, first thing is you have to have a source, excitation signal, the first process. Excitation, then there will be an emission of course through the fluorescent process from the sample, there will be an emission in the fluorescence way by fluorescence method.

And then finally you have to detect this fluorescent signal that is it. For this what you have typical components is that you have this source, light source. What I said is that this excitation wavelength is very particular, so fluorescence effect. So, fluorescence microscopy takes advantage of fluorescence effect. So, for this this excitation, emission both these process, entire fluorescence process, it is an energy sensitive process.

So, the wavelength of excitation voltage and wavelength of emission voltage is very much fixed and particular. So, the light source excitation voltage that you use, excitation filter or excitation wavelength that you use that is sensitive to the kind of material you are having, you are testing or kind of material that is going to give you fluorescence ultimately. So, that is why this excitation generally you have a normal light source, xenon arc lamp or mercury vapor lamp you can have them.

Otherwise, you can also these days it is more common to use high power lasers or LEDs because they are inherently the way LEDs are generated, the way laser beams are generated, inherently they have a very narrow spread of wavelength. You can control

that which wavelength you want. Which wavelength of light you want that you can very well control if you use LED or a laser.

Xenon arc lamp, mercury vapor lamp you do not have that much control. After that you have an excitation filter. What does this filter do is that basically it only allows certain part of that lights depending on the wavelength. So, particular wavelength is only allowed through here and basically the rest of the things is absorbed. So, this works like an aperture, what we saw in condenser lenses and also this works like an aperture.

But in this case the aperture, the kind of it cuts down the light source depending on the wavelength. So, it is only allowing certain wavelengths to pass through. The next one you have is called a beam splitter or dichroic mirror. What does this dichroic mirror do basically, again it reflects or it reflects back this incoming excitation signal, it puts it back through this objective lens it is putting it to here, it is focusing basically finally on the specimen from here.

Then normally in fluorescence microscopy what happens is that we will come to that that mode. So, now the emission signal that this material develops, the emission signal also passes through the same path, same objective with a different wavelength. So, then what is the dichroic mirror will do is that now it is so sensitive that it will only allow the emission signal to pass through.

So, it will reflect the excitation signal, so it will not allow the excitation signal to go anywhere else to transmit through, but it will only allow the emission signal to fall to go through here. So, this entire fluorescence microscopy is all about this wavelength, which wavelength to pass through, which one will go, which one will come back and all those things.

So, finally once you have the emission signal coming out of this dichroic mirror and then you have the camera, you have this beam splitter and all, you can either take it in a CCD camera or look at in the eyepiece that is all. Normally the fluorescence microscopy generally works or almost always I would say, I have not seen it, almost always fluorescence microscopy works in the transmission mode.

I have not seen it. Because of this entire component and all it is very difficult to find out fluorescence material in reflection mode, it is difficult to configure at least that is not something I know of, I am aware of. And fluorescence microscopy of course is very useful technique for biological samples as I said right in the beginning. Genetic cell biologists they use fluorescence microscopy very often.

And because here the emission profiles, spatial resolution and high specificity of fluorescence investigation this is. There are known fluorophores. The materials, molecules which generate fluorescence effect under certain excitation wavelength and under the kind of emission wavelength they generate. So, this combination is well known for many of the molecules and this fluorescence microscopy, the biologists take advantage of this.

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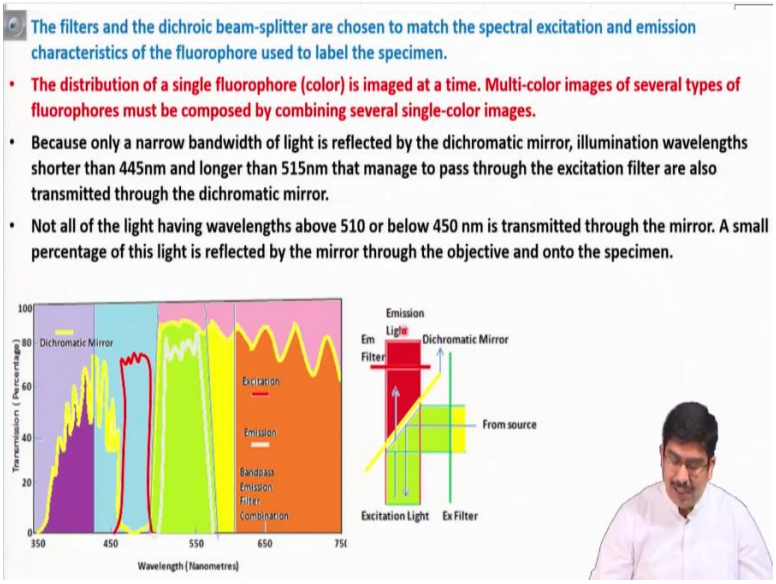

Now, as I was saying this dichroic mirror is very, very important here. So, dichroic mirror what it does I already explained. The dichroic mirror basically it will not allow the excitation voltage or excitation lights or excitation wavelength or excitation light, it will not allow excitation light to transmit. It will reflect it back to the specimen here, but once the emission light is there this dichroic mirror will be transparent to the emission light. So, that is what the dichroic mirror basically does.

And for that if you look at here the wavelength versus transmittance that is what very important. So, the dichroic mirror, the excitation filter everything is sensitive to the material that you are finally going to check under fluorescence microscopy. What kind of

wavelength you want, what kind of a dichroic mirror you will be putting there which will allow only certain part of the light to transmit, certain other part will be reflected, all of this is very crucial and all of this is very fixed.

So, if you look here the dichroic mirror basically the transmittance of the dichroic mirror for excitation signal is very less, so it will not let the excitation signal to transmit through, whereas again the transmittance for the emission signal in this wavelength for the dichroic mirror is very, very high. So, all the emission signal will pass through. They will not reflect it back to the source, they will just go through this path.

So, they will follow a completely different path altogether. So, that is how the fluorescence microscopy, this dichroic mirror basically splits the beam or basically makes the two different, this excitation light and emission light, it makes it to travel into different paths. So, this dichroic mirror this is a very special component and so as the excitation filter and emission filter.

So, emission filter is also very, very important here because if you look this emission filter will again filter out. The point why emission filter is very important is that dichroic mirror will not be able to, not everything in the excitation light will be reflected, some part will definitely be transmitted because we are dealing ultimately with a ray. So, that part will be again chopped up by this emission filter.

It will only allow a certain wavelength and emission light again, if you remember I said the emission light will also be having a large spread in the wavelength particularly if it is a phosphorescence effect. So, it will have a large spread. So, this large spread again will be filtered out by the emission light, so it will just allow one particular wavelength so that that finally I will get one particular color according to that material which is generated according to the molecule which is generating this fluorescence effect fine.

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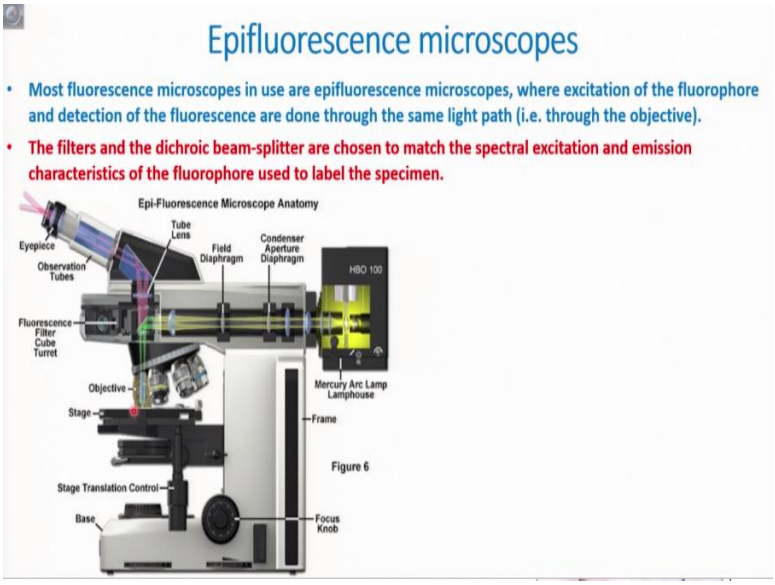

So, what is epifluorescence microscope. Now, this kind of microscopes are called epifluorescence microscope. An epifluorescence microscope basically is exactly the same. You have this emission voltage, emission signal and emission light and excitation light. Both of these is passing through the same objective, right. So that is why they call epifluorescence.

So, we have seen other epi-illumination bright field, they are also the reflected mode. The incoming signal, the incoming light and the reflected light travels through the same objective lens. So, there are also it is kind of an epi-illumination configuration, here also the same thing. The fluorescence microscopy almost invariably works in that epifluorescence mode so that this excitation light and this emission light they both travel through the same objective.

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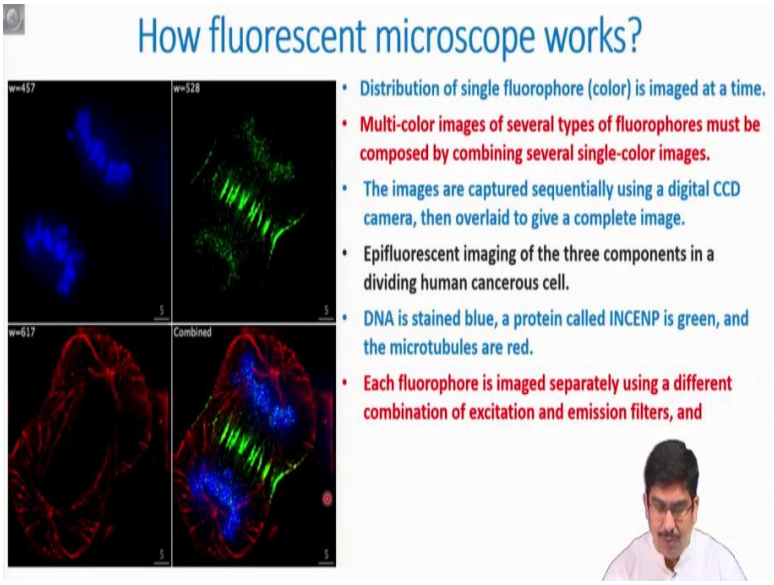

Now, how fluorescent microscope basically works finally? So, fluorescent microscope is first I have something which is producing a fluorescence effect. So, these are called generally fluorophores. So, it is producing a fluorescence effect in the beginning and then I am getting end signal, emission signal out of that which is again particular. There is a particular wavelength for that which corresponds to particular color.

So, for one type of molecule, one type of material which is showing fluorescence, one type of fluorescence effect I will get one type of color that is one. If I have multiple such fluorophores in the materials, either naturally or possibly I have generated that, I will come to that. If I have multiple such fluorescence in the material which is producing fluorescence effect, then I can do this entire.

And if you know as I said that excitation light and emission light they are coupled. So, for getting another fluorescence effect, another molecule to show fluorescence effect I have to possibly go to another wavelength in the excitation there that is where the excitation filter is so important. So if I do that I get another color, I image another regions and I go on and do that for another one.

So, these three and then finally what I do I just superimpose these 3 images. And I get the final image where I could see 3 different regions with 3 different colors, which are produced by the fluorescence effect. And this one that is showing here is basically a human cancerous cell biological sample, here the DNA, this part is the DNA which is stained in blue by a protein, it is not a real, the DNA does not show that color.

It is basically there is a staining agent, it is a protein which goes there and binds with this so that is producing this fluorescence effect okay. So, the DNA is showing the blue color, then another protein is showing the green color and finally the microtubules are showing the red color all of this separately, and then finally they will be showing this image.

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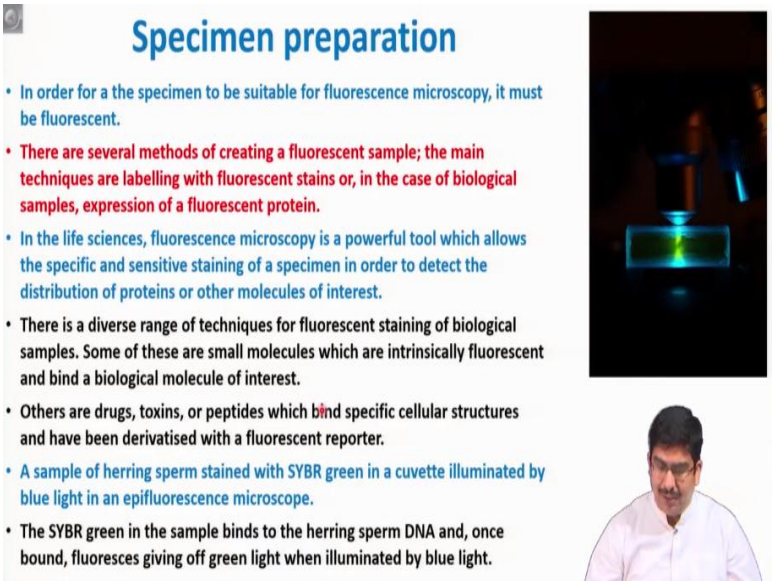

So, of course as I said sample preparation is very, very important here because naturally the biological materials do not show this fluorescence effect. You have to artificially stain them, you have to artificially add certain fluorophores or certain material which creates this fluorescence effect. So, those kinds of materials will go bind with the biological samples and produce this fluorescence effect.

And for those material for the staining material, you already know that what is the excitation light, what is excitation wavelength, what is the emission wavelength. So, you basically have to know that and that also you have to know that which protein goes and binds to what kind of a sample which protein of your sample. So, you must know this and then you basically stain your material.

Stain the biological material with a suitable particular fluorophore and then that is how you prepare your sample. So, sample preparation in fluorescence microscopy is very, very important. You must know a priori what kind of specimen you are going to check, what proteins are there, what you are going to expect to see, then what kind of a staining

agent I should add, what that staining agent go and bind to what, for DNA as I said, this is very, very specific.

So, there are certain proteins which goes and binds the DNA only. So, that kind of a protein you have to stain it and finally then you can put it for this fluorescence microscopy. And that is what it is shown a herring sperm stained with some kind of a protein here. And this stained, as I said these biological specimens mostly they are proteins, which are small molecules.

Some of these are small molecules, which are basically fluorescent and bind to biological molecules. You can also artificially generate that by using some artificial drugs, which has pretty much the same structure at the proteins or peptides and they also go and bind to cellular structures and then they can give you the fluorescence effect.

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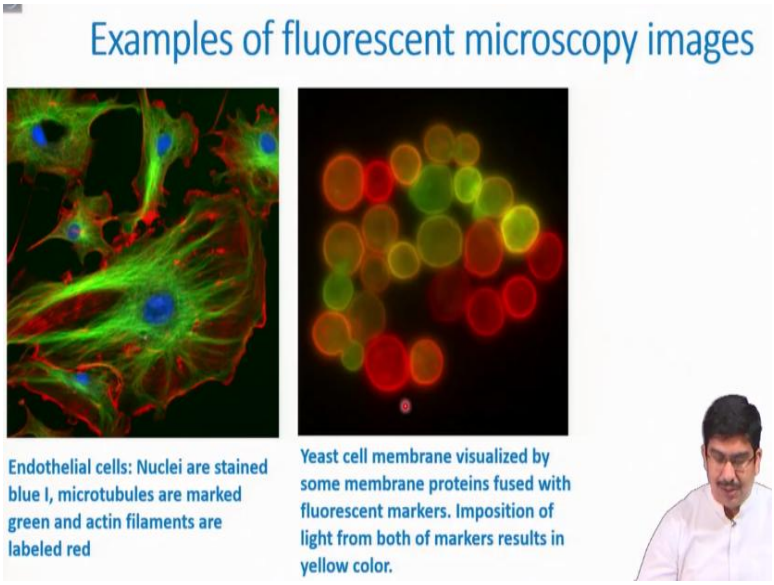

Some examples of this fluorescence microscopy images; you can see the beautiful images of some cells and then the nucleus gets one kind of a color the other proteins are getting some other different kinds of colors. This is another example of a yeast cell membrane that is visualized. So, you can see the membrane is showing a different color and then the material within. So, this is also kind of a staining.

So, the staining causes the membrane to show the red color and then the inside one that is showing some other color. Remember one thing that in the fluorescence microscope, again you have those absorption all those effects which is there in bright-field

microscope that is still happening there also. So, you will have this contrast generation even between the same

So, ideally if it is only a yeast sample, then everyone should show, only showing the fluorescence material and stained by a particular type of protein, then all of them should show you the same contrast that is not happening because on top of the fluorescence you have still the bright-field effect. That means, you still have the scattering effect and the scattering effect will make certain regions produce a different contrast for different yeast cells still.

And these different colors is sometimes caused by superimposition of different type of lights because if you have multiple such cells on top of each other, then there will be some superposition of these colors.

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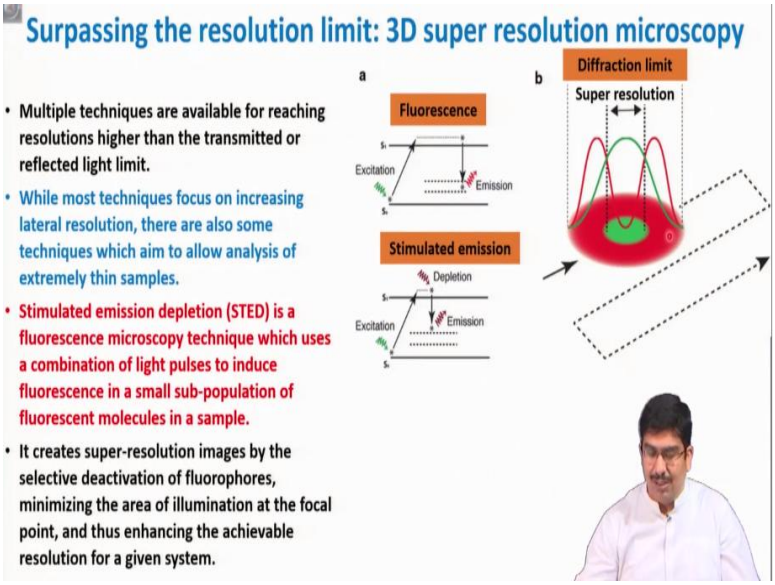

And final thing that I wanted to discuss very quickly is surpassing the resolution limit. As we said that the diffraction, basically because of the diffraction effect we have a resolution limit which is around 200 nm or little less 150 nm. If you use some special lights, then around 150 nm is what we can go with the optical methods.

Now, the point is these optical methods using some tricks you can even surpass this diffraction limits and the best way to do it is to reduce the size of the beam or reduce the size of this incoming signal. Where from this incoming signal is coming you can reduce that size so that now you are generating a light from a region in the specimen which is

even lower than the diffraction related limit. So, it is not depending on the incoming signal.

This diffraction related limit is set by the incoming numerical aperture of condenser lens and objective lens. From this material if you somehow can produce lights from a very tiny place, very small place which is even smaller than the diffraction limit, you can improve the resolution and this is what is possible by fluorescence effect in particularly one technique which is called STED, stimulated emission depletion technique, there exactly this is what is done.

The region which is producing the fluorescence effect is even smaller than the diffraction related region, usually some molecules very tiny molecules. So, now you are getting the emission light, you are getting the signal from the specimen from even smaller regions. So that is why the resolution is now increasing and that will help to surpass this diffraction-limited resolution of these materials and that is how you can see.

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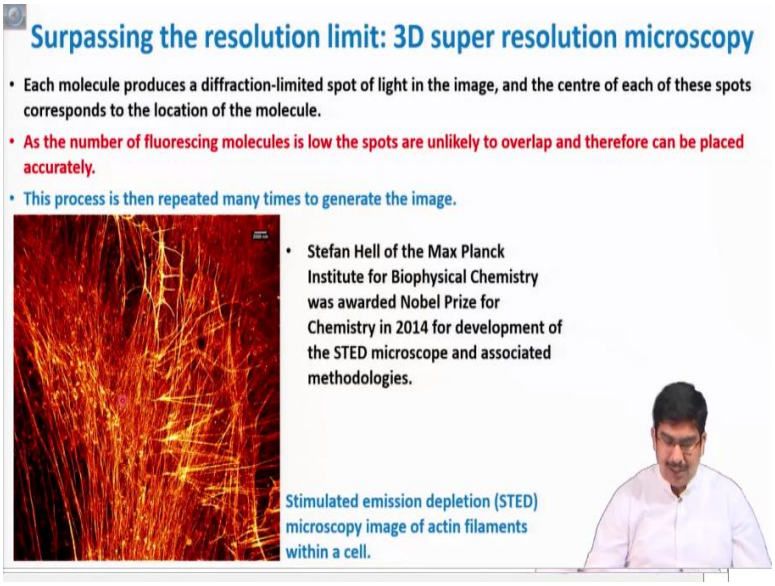

This is one image of actin filament within a cell and you can look at the nice resolution here and you can look at this scale bar. So, you are really probing something in the nanometer range with an optical technic, of course you use a laser-based technique here. So, that also helps to improve your diffraction-related resolution, but more than that you basically use a fluorescence effect here.

You have fluorescence fluorophore molecules and selectively you basically excite those fluorescence molecules and that is how you can generate a source of signal which is far apart further apart and you improve the resolution. And the discovery of the STED microscopy was so important and the discoverer Stefan Hell of Max Planck Institute he was awarded a Nobel Prize in Chemistry in 2014.

And the importance of that is basically because biologist for a very long time they were looking for a technique which can give them resolution in the nanometer range or even lower than that which the material scientists very regularly can get such kind of a resolution using electron microscopes, which we will be discussing in subsequent classes. They can very easily get such resolution.

They can achieve that resolution just by reducing the lambda value the wavelength. But one problem is the electrons typically destroy the biological samples. Because of their very high energy, they completely destroy biological specimen which are basically carbon. So, they burn electrons, basically burns the carbon and the kind of if you want to go to that kind of resolution, you have to use a TM and those TM will just burn the biological samples.

So biological samples very difficult to do it on electron microscope. So, biologists who are looking for a technique which is optical based which will not damage their specimens but still can give them nanometer range resolution, and this STED is one of the solutions they could achieve that is why it was so important. And that entirely depends on this fluorescence method.

So, with this we are ending it here with this optical microscopy part and from the next class onwards we will be moving to electron microscopes. See you there. Bye.