Spectroscopic Techniques for Pharmaceutical & Biopharmaceutical Industries
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Lecture 28
Contrast in Microscopy, Fluorescence Microscopy

Hello students, welcome back to lecture 28 of this course. In the last lecture, I started discussing about microscopy. We discussed about four very important concepts in microscopy, one is your magnification, the second is resolution, and the third is your illumination and then we started discussing about the fourth, which is contrast. There are other methods, I started with phase contrast, there are other methods to increase the contrast in microscopy, and I am going to discuss one of such method and that is polarization.

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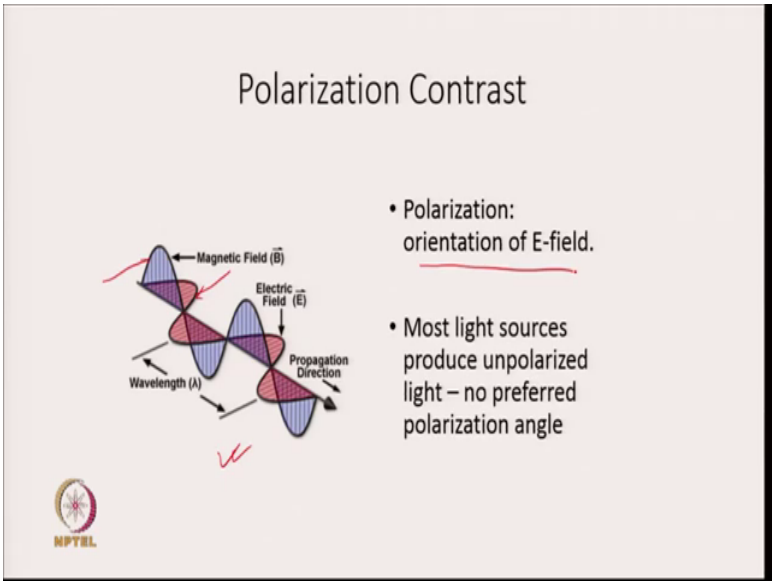

So, contrast can be achieved with the polarization. So, first thing which we need to know is what is polarization. This is orientation of electric field most light sources produce un-polarized light and what does that mean is that it does not have preferred polarization angle. So, here is your electromagnetic wave and as we discussed during our initial lectures that there are two fields, magnetic and electric field which are orthogonal to each other and here what we are concerned about is orientation of electric field.

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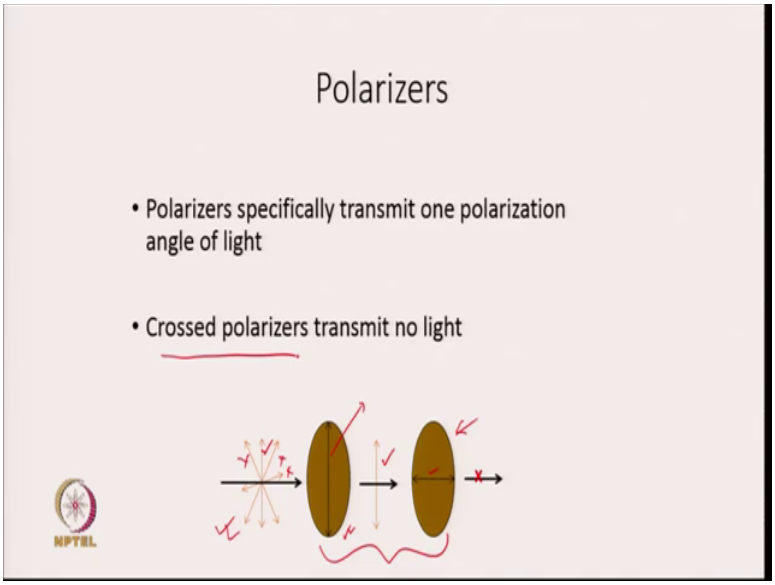

So, but polarizers does, they specifically transmit one polarization angle of light, so if you take normal light what happens is there is no preferred angle off that is what we talked about here, no preferred angle, no preferred polarization angle, so ordinary light is a mixture of polarized light with at different angles, polarized light at different angles. When you pass this through a polarizer, what it will do is, it will transmit only one polarization angle of light.

So, here you see if I take a, you know, polarizer whose axis is like this vertical, then it will only allow this vertical light to pass through, to pass through other lights are blocked, other lights are blocked. And now, suppose I take another polarizer, the axis is horizontal, axis is horizontal, then what it will do is, it will not allow this light to pass through, to pass through, so if you have a combination of this two polarizers, one is vertical polarizer followed by a horizontal polarizer, then there will be no light which will transmit and this kind of polarizers are called crossed polarizer.

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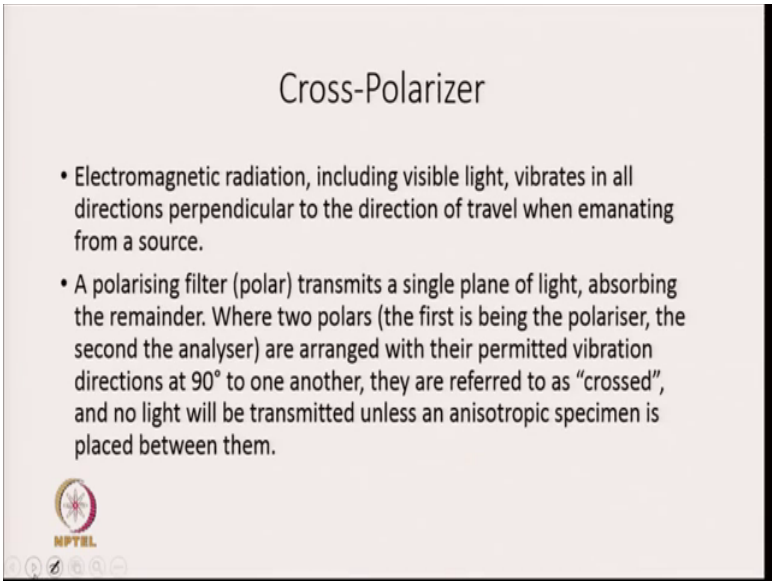

So, electromagnetic radiation including visible light vibrates in all direction perpendicular to the direction of travel when minuting from a source that is what I discussed, that before passing the polarizer your electromagnetic radiation vibrates in all directions perpendicular to direction of travel. What polarizer filter does? It transmit a single plane of light, single plane of light absorbing the remainder and if you use vertical polarizes polarizer.

It will transmit a vertical plane of light where two polarizer are arranged with their permitted vibration direction at 90 degree to one another, they are referred as cross polarizer and no light will be transmitted until an anisotropic specimen is placed between them. If there is no anisotropic specimen, then no light will transmit.

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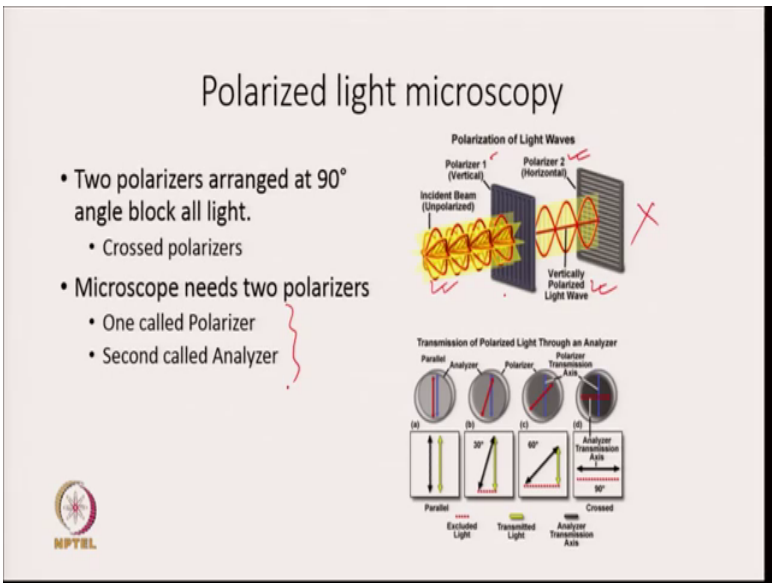

So, in polarized light microscopy what we do that we use two polarizer arranged a 90 degree angle to block all light. So, that is what you can see that this is your incident beam which is unpolarized, when it pass through a polarizer one, then you have vertically polarized light wave and when it pass through the polarizer 2, which is horizontal then after that there will be no light getting transmitted. Microscope needs two polarizer, one is called polarizer and another is called your analyzer. Crossed polarizer, I already discussed that crossed polarizer is two polarizer arranged at 90 degree but microscopy needs two polarizer, one called polarizer and second call analyzer.

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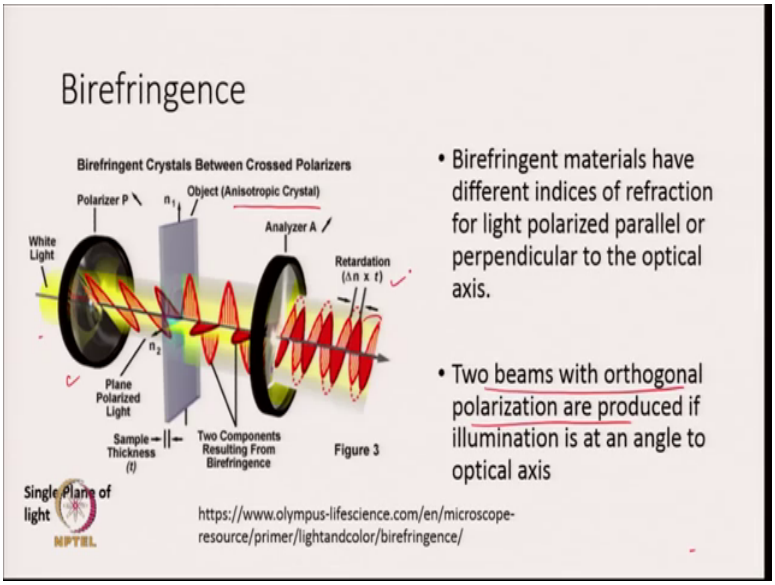

Here we use by refrigerants, by refrigerant materials are quite unique, they have different indices of refraction for light polarized parallel or perpendicular to the optical axis. So, when we pass white light through a polarizer it gives you single plane of light, it gives you single plane of light, now if we put analyzer and what we expect that there will be no light, we should go across this analyzer.

But what we did is, we put by refrigerant material which is basically an isotropic crystal. It has property that it has different indices of refraction for light polarized parallel or perpendicular to the optical axis and so what you will get is, two beams with orthogonal polarization. If we put illumination at an angle of the optical axis, if it is a long polarizer axis, then this will not be seen.

Only when you put it at an angle to the optical axis, then two beams with orthogonal polarization will be produced, two beams with orthogonal polarization will be produced and when it pass through analyzer, there will be a path difference between the two rays, one is

known as ordinary ray, another is known as extraordinary ray and their retardation is given by this formula delta n into t.

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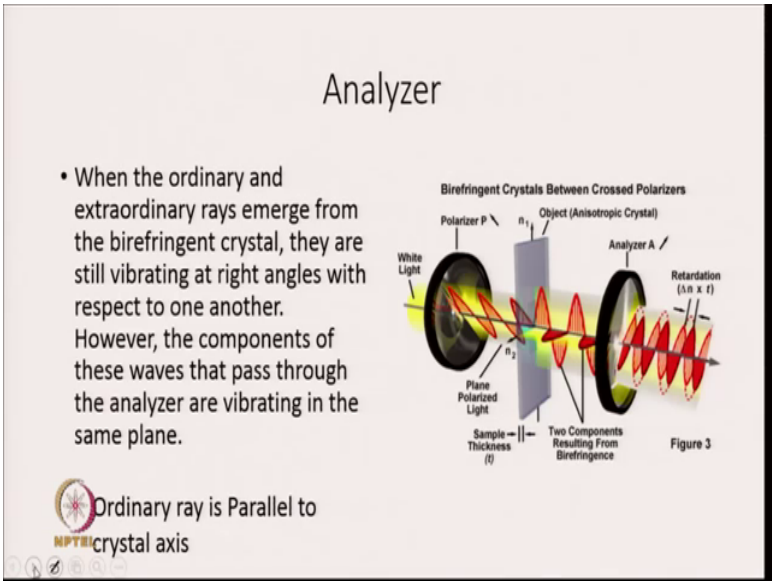

So, when ordinary and extra ordinary rays emerged from the by refrigerant crystal, they are still vibrating at right angles with respect to one another, so after passing through this there will be two orthogonal rays, one is called ordinary ray, another is called extra ordinary ray, and they are at 90 degree to each other. However, the components of this wave that passed through the analyzer are vibrating in the same plane.

So, these are the, these are the waves, these are the component of ordinary and extraordinary waves, they are vibrating in the same plane, but with a path difference and that path difference leads to retardation, that path difference leads to retardation.

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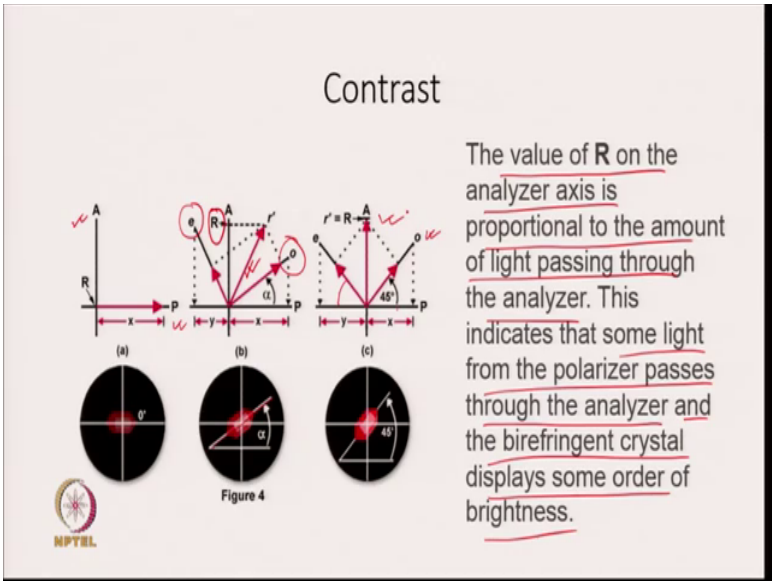

Now, let us understand it much more clearly, this is your XY diagram. On y axis this is y axis there is A which denotes analyzer axis and here is t which denote polarizer axis, polarizer axis. When you have the by refrigerant crystal not making any angle with the axis, then what will happen, that all light will go through polarizer and we cannot see anything on the analyzer, nothing will pass through analyzer.

But, if suppose we put at angle alpha, if we put this crystal at angle alpha, then what will happen that a ray will be, the analyzer will pass a ray along this axis and that is known as ordinary ray and there will be another ray, which will be coming out is a perpendicular to this ray and that is known as extraordinary ray, they are orthogonal to each other, they are orthogonal to each other and resultant of these waves is shown here, resultant of this wave is shown here.

And the component of this resultant on analyzer is given by R, analyzer is given by R and the value of R on the analyzer axis is proportional to the amount of light passing through the analyzer. So, value of R on a analyzer axis is proportional to amount of light passing through the analyzer, this indicates that some light from the polarizer passes through the analyzer and the by refrigerant crystal displays some order of the brightness.

If this crystal, by refrigerant crystal makes an angle 45 degree, then your ordinary ray will make 45 degree with this axis positive P axis, whereas extra-ordinary ray will make 45 degree angle with negative P axis and the resultant will be maximum, the resultant will be maximum.

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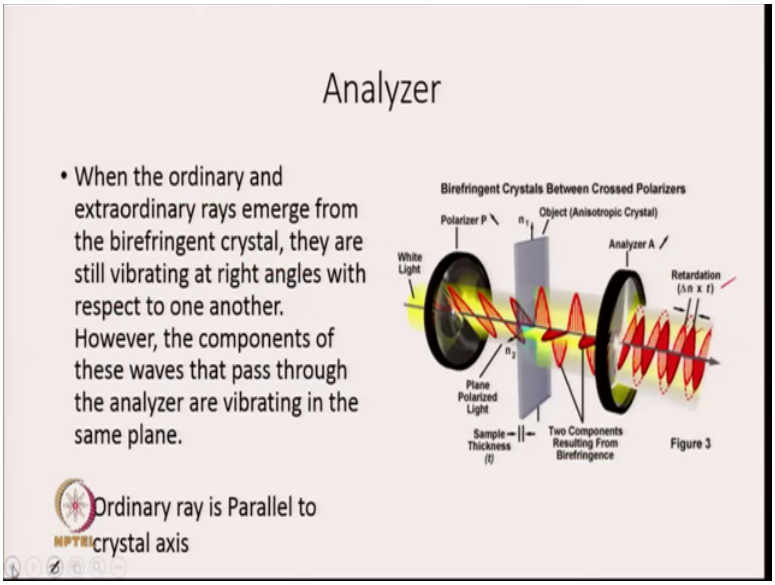

So, if you look at this what we told that there will be a path difference if this light passes through analyzer A, these two extra ordinary ray, an ordinary ray passes through the analyzer A, they are going to be in same plane, but with a path difference, and the path difference or retardation is given by delta n into T.

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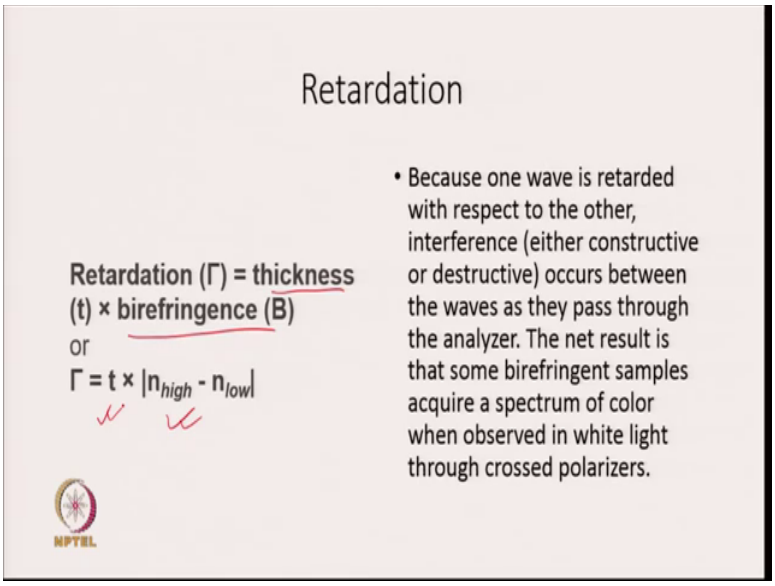

And that is what is written here, retardation is thickness into by refrigerants, which is basically the refractive index difference between high and low and t denotes thickness. So, because one way be retarded with respect to other interference, either constructive or destructive, occurs between waves as they pass through the analyzer and the net result is that

some refrigerant samples acquire a spectrum of color when absorbed in white light through crossed polarizers and this is how contrast is generated in polarization microscopy

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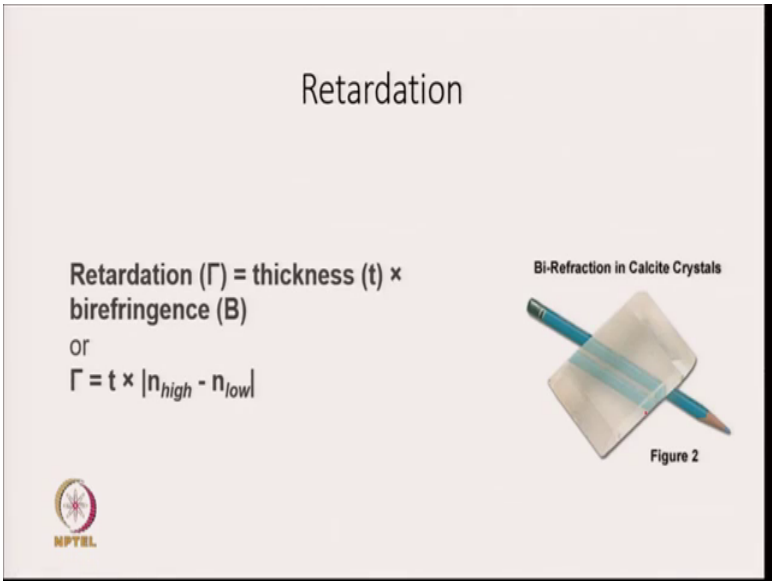

And now you can see that we take a pencil and put a, by calcite crystal on it, you will see the two figures.

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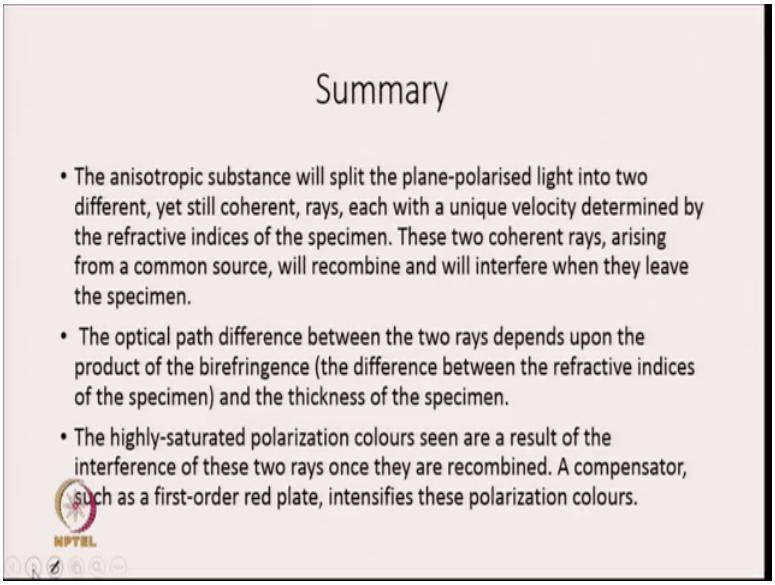

In summary the anisotropic substance will split the plane polarized light into two different, yet is still coherent rays, each with a unique velocity determined by refractive indices of specimen. These two coherent rays, arising from a common source, will recombine and interfere when they leave the specimen. The optical path difference between two rays depend on product of by refrigerants and thickness of the specimen.

And the highly saturated polarization colors seen as a result of interference of these two rays once they are recombined. A compensator such as first order red plate, intensify this polarization colors and that is how contrast is generated in polarization microscopy.

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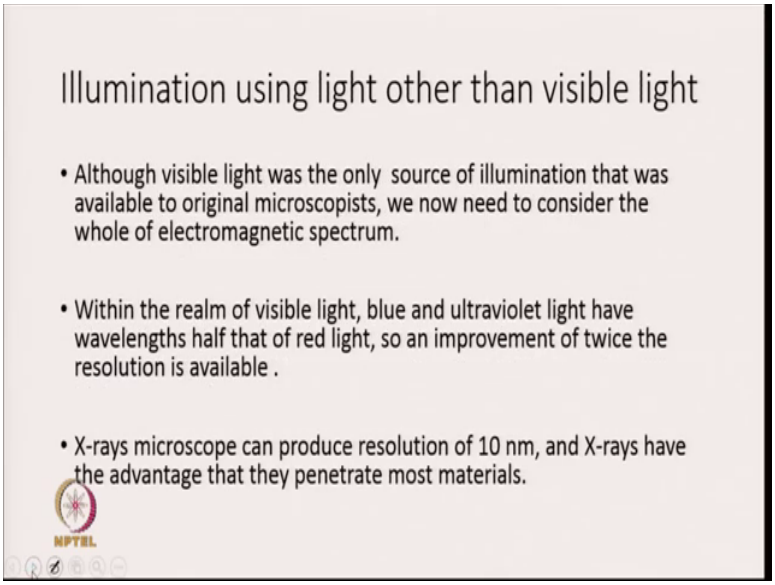

Till now we have seen the illumination in microscopy being done with the visible light. But illumination can be done using lights other than visible light. We can basically employ the whole of electromagnetic spectrum. Even in the realm of visible light, blue and ultraviolet light have wavelength half that of red light, so an improvement of twice the resolution is available. D is proportional to lambda and if D is low then resolution is high.

So, if I, half the wavelength, if I half the wavelength D will be half and, thus resolution will be higher. If you go to even a smaller lambda, for example, if you go to X-rays they can improve the resolution and so X-ray microscope can produce resolution of 10 nanometer and there is another advantage that X-ray can penetrate most material. So, keeping this in mind Kohler also thought about developing a microscope which is based on UV light, since UV light has smaller lambda than visible light.

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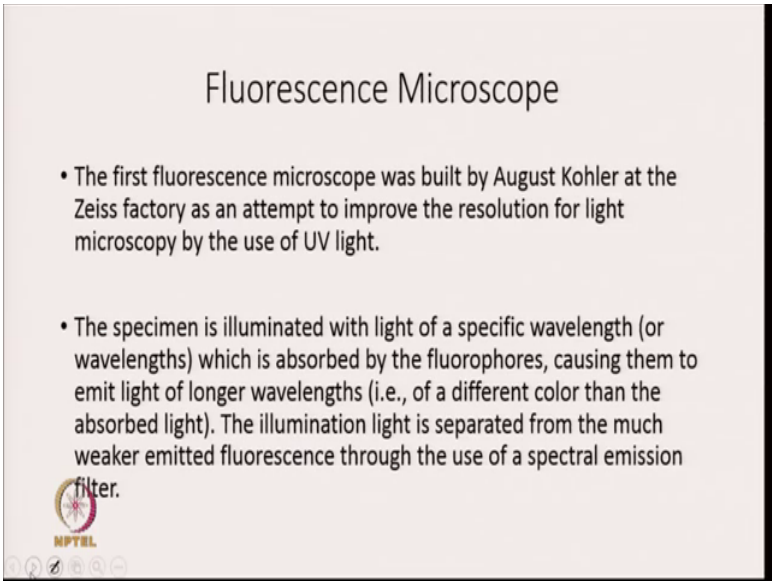

So, first fluorescence microscope while built by Kohler at the Zeiss factory as an attempt to improve the resolution for light microscopy by the use of UV light. In that what he did is, the specimen is illuminated with light of a specific wavelength, which is absorbed by the fluorophore, causing them to emit light of longer wavelength. The illuminated light is separated from the much weaker emitted fluorescence through the use of spectral emission filter.

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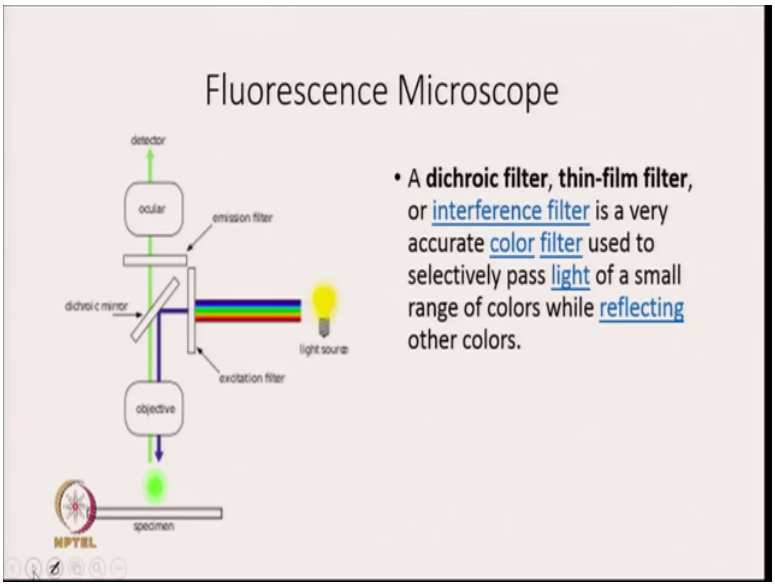

And this is your schematic of a fluorescence microscope, so what it has been done is, light comes from this source. It is reflected by dichroic mirror, it is reflected by dichroic mirror. It comes passes through objective and then it is absorbed by a specimen. A specimen emits the

light and it passes through objective, then it can pass through dichroic mirror. The reflected light will not pass through it, and so there is importance of this dichroic mirror.

This dichroic filter which is a thin flim filter is a very accurate color filter used to selectively pass light of a small range of colors while reflecting other colors, so other colors will be reflected and only the emitted light will pass through this dichroic mirror to detector, to detector and that is the principle of fluorescence microscope.

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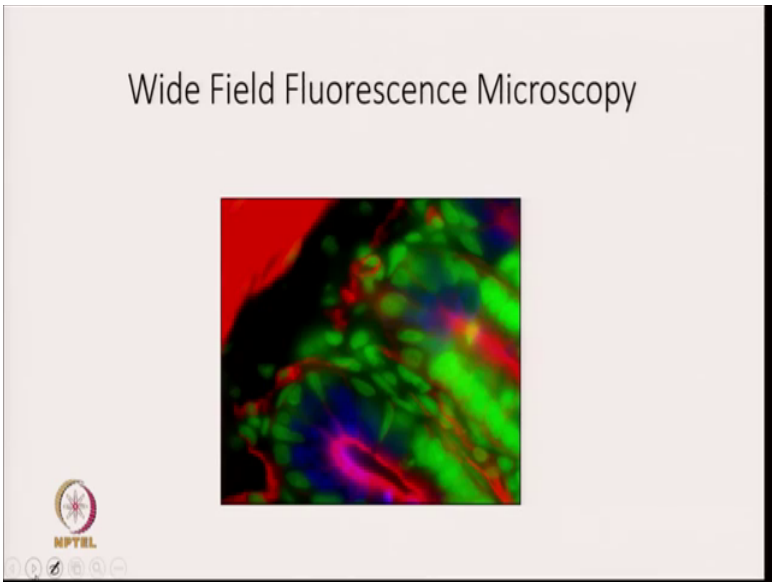

There was one problem that this wide field fluorescence microscope, so wide field means, if I am trying to take the image of every part of the specimen at a same time or we are trying to take the picture of a specimen, picture of a specimen. As a whole and what you will get is, you know, blurred picture, blurred picture, and so if you want to take picture of a specimen at one time, then there is a, there will be a problem of your higher resolution and that is one of the main disadvantage of wide field fluorescence microscopy.

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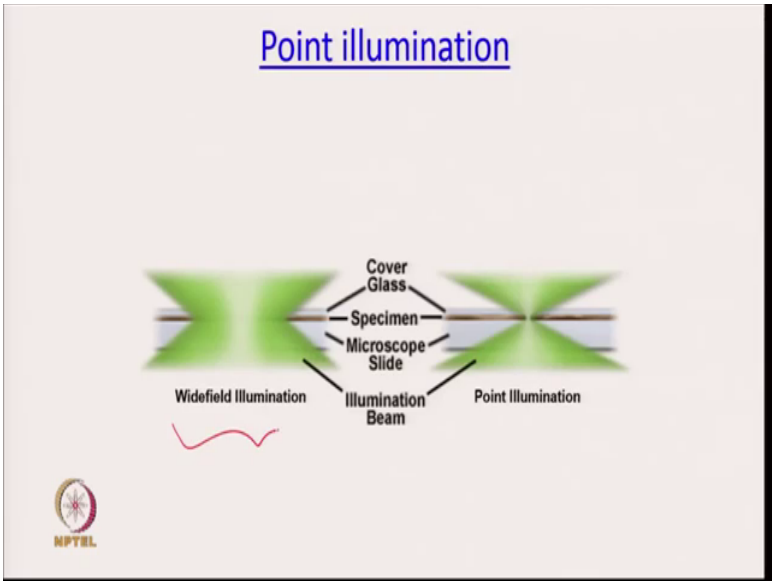

So, wide field illumination is just like this and that leads to blurriness in the images. So, what people come across is what is known as point illumination. So, in point illumination, you can remove a blurriness.

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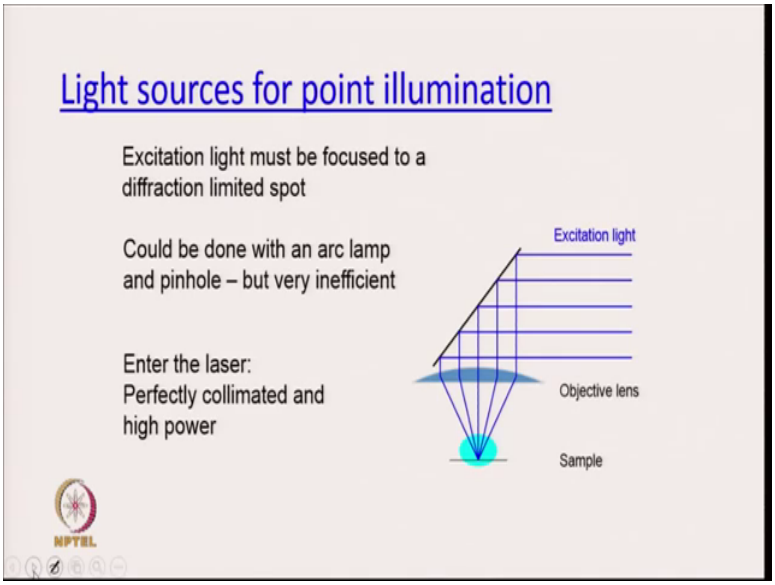

So, let us discuss this. So, what happens that in the point illumination what you do is, you take excitation light and you try to focus to a diffraction limited spot, so one spot, we try to focus it to a diffraction limited spot and if you want to do with a arc lamp and pinhole, you will be able to do that, but it will be very inefficient. And so, you can use perfectly collimated and high-power if you do that you will get your point illumination.

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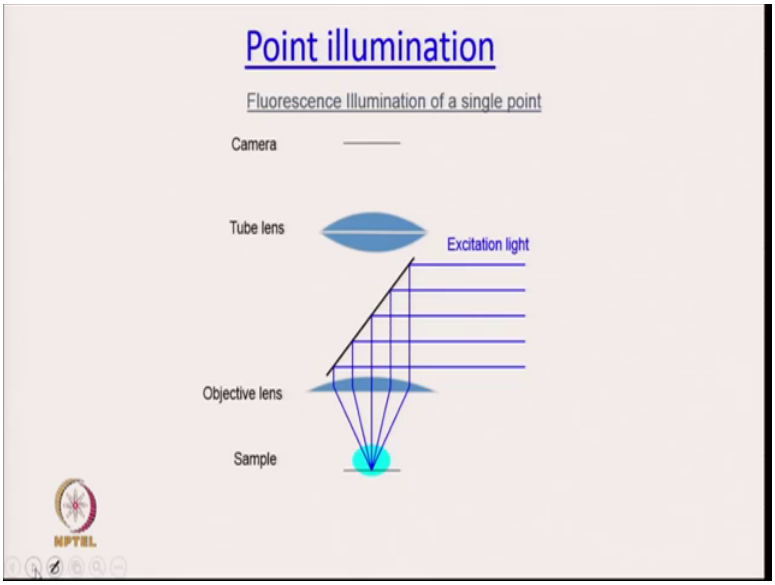

So that is what we is done, fluorescence illumination of a single point, what you do, you put excited light and if it does you put an objective lens, and since it is coming parallel, and it will your focus, the light on a single point, it will focus the light on a single point and that is how you get a point illumination.

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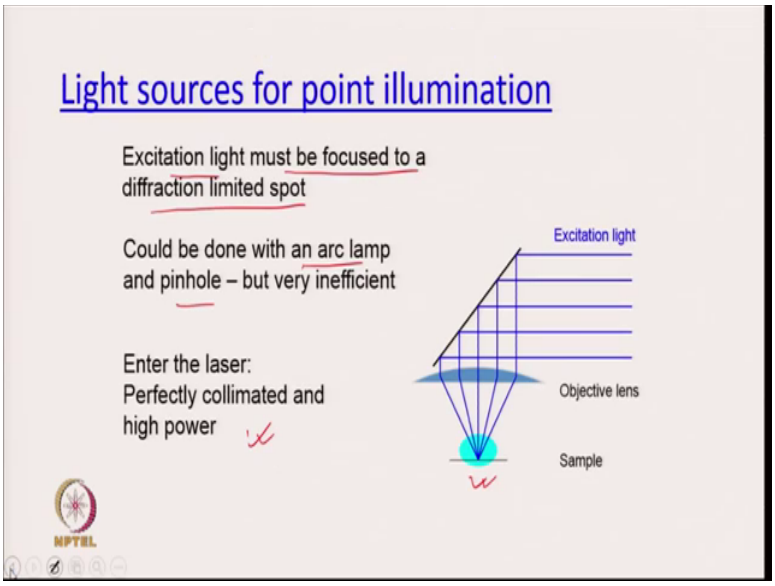

So, if you remember here that what we told that we need to get at one single point.

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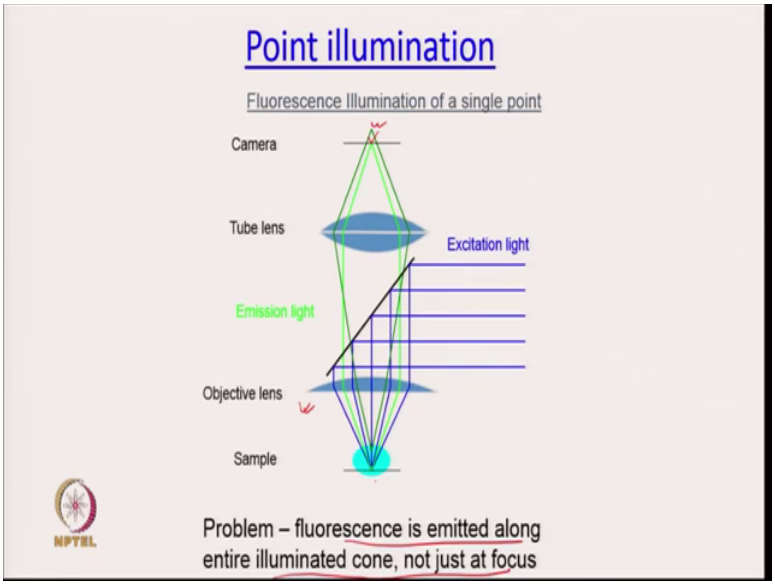

So, this is your point illumination. And once this sample or specimen absorbs the light, it will, if it has the right fluorescence property, it will give you this emission light, emission light and if emission light goes like this here there is turbulence, it will again focus to camera and then one point picture, one point picture on a spot, on a specimen will come as image at one point.

But there can be a problem, what can be the problem, that if suppose light start from here, then you can get image at this point, so there will be a blurriness, there will be a blurriness, so fluorescence is emitted along entire illuminated cone, not just at the focus, just at the focus.

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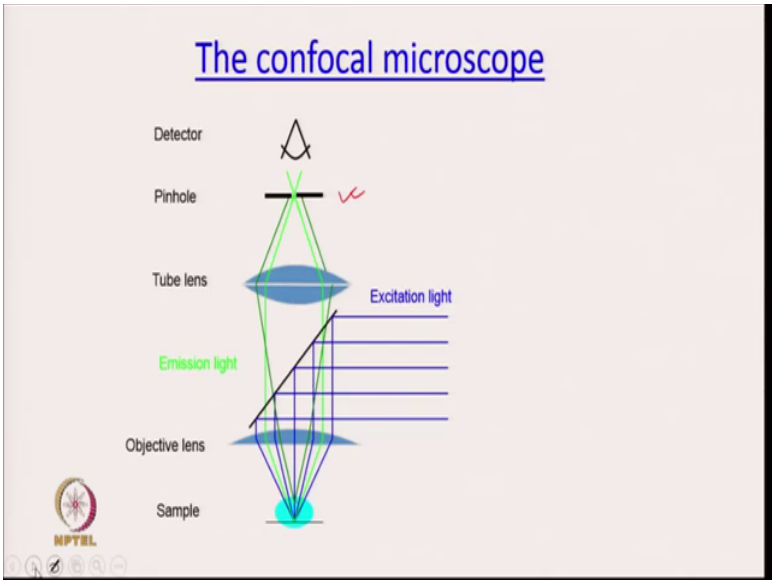

Now, comes the confocal microscope, so to avoid this blurriness what has been used is a pinhole, a pinhole is used. Now, what will happen, if you place a pinhole between tube lens

and detector what will happen that emitted light from this point will pass through this pinhole. But, if it comes from some other places, then you see this is stopped emitted from, the light emitted from this point is a stopped here, stopped here and that is how you can avoid the blurriness in the image.

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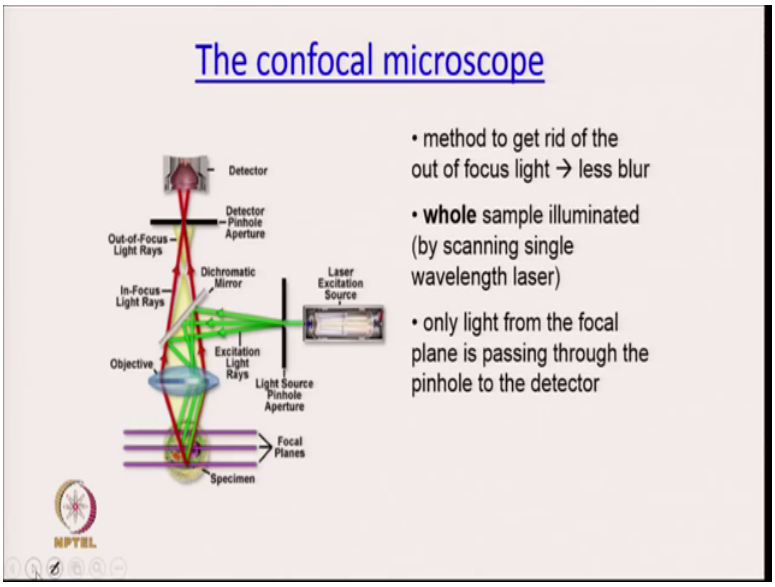

So, confocal microscope is a method to get rid of out-of-focus light and so less blur. Whole sample is illuminated by a scanning single wavelength laser, only light from the focal plane is passing through the pinhole to the detector. So, you can see this is a much better picture, so here this is the laser excitation source, then you put a pinhole aperture here also, and that allows you to pass the light of single wavelength and it goes, it passes.

When it strikes at different focal plane, then it will emit light, and that can pass through your pinhole out-of-focus light, from other places. Will be stopped at this pinhole and that is how you get a much enhanced resolution of the picture.

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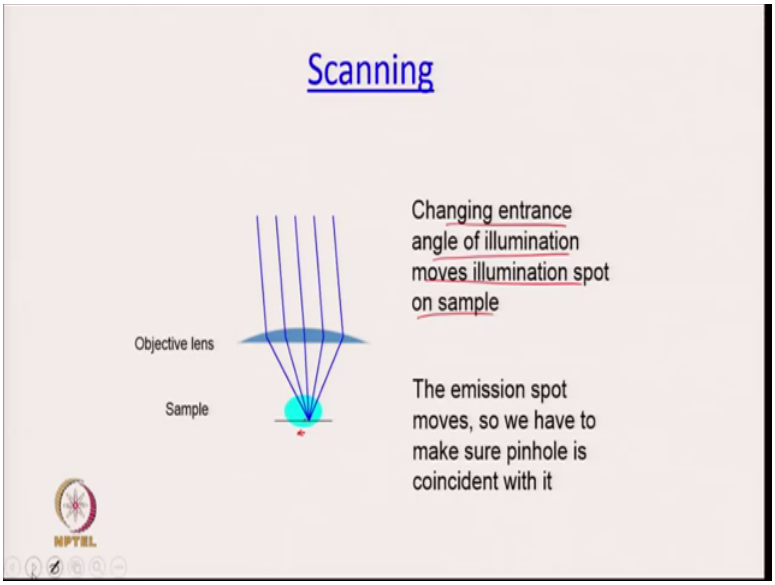

Now, what you do is, you can change entrance angle of illumination which will move the illumination spot on the sample. So, till now we have got only one point, picture of only one point, but if you want to get the picture of whole specimen then what you do is, you change entrance angle of illumination and that will move illumination spot on the sample. And that is done by taking, you know, so what you do is entrance of, entrance angle of illumination is changed and now this point from this point to shifted to this point.

Okay, so the point of focus will change, point of focus will change, so now you are getting the image of the second point on the sample or a specimen. Now, if you take this kind of ray, then you will get the image of this point, image of this point. And so emission spot moves so we have to make sure pinhole is coincident with it, so we have to make sure that pinhole is coincident with it, so you are moving this entrance angle of illumination.

And then you are shifting your pinhole to get a much better picture of each point, of each point. And that you do in raster fashion, so you take picture of one point, two point, three point, four point, five point, six point, and then you go in different planes and you take picture.

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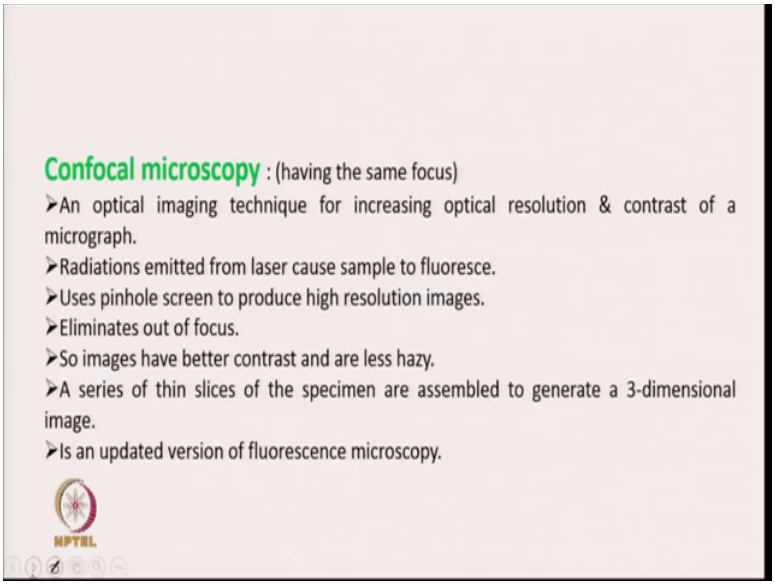

So, in confocal microscopy it is a basically an optical imaging technique for increasing optical resolution and contrast of a micrograph. Radiation emitted from laser cause sample to fluoresce. Now you are using pinhole screen to produce high resolution images. It eliminates out-of-focus rays, image has better contrast and they are less hazy. A series of thin slice of the specimen are assembled to generate a 3-dimensional image and basically it is a, an updated version of fluorescence microscopy.

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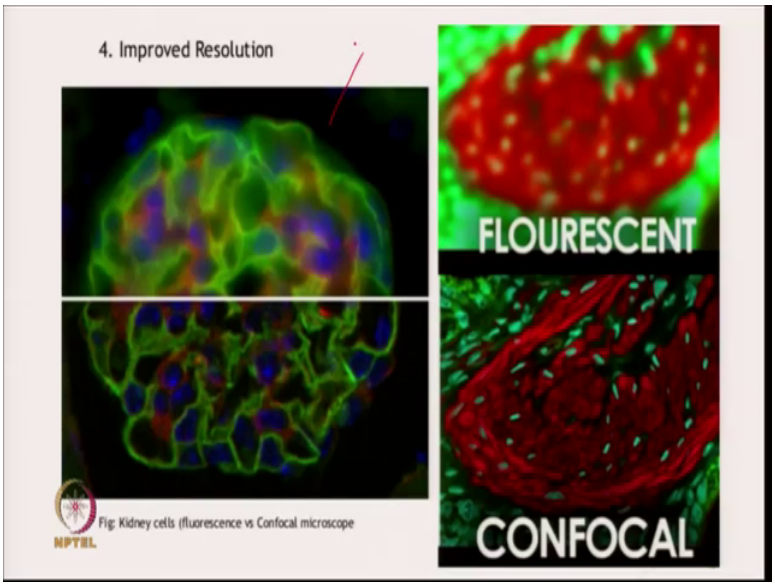

Now, you can see this is wide field microscopy versus your confocal microscope, picture are more clear, less hazy. These picture are more hazy, these picture are less hazy and a better contraction a contrast has been achieved. Similarly, you can look at this picture, this is taken

using wide field microscopy and this is taken using confocal microscopy, and this is picture of kidney cells and you can see what is the advantage of using confocal microscopy.

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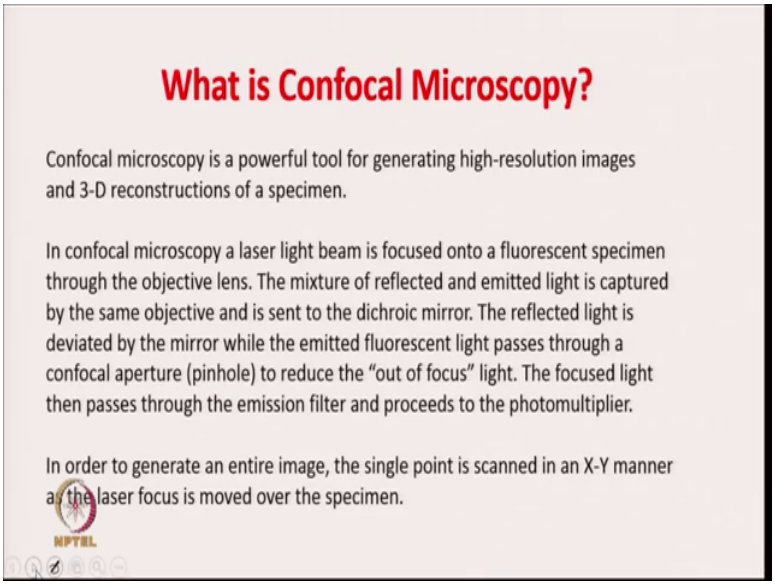

So, in confocal microscopy you are generating high resolution images and 3D reconstruction of a specimen. Again what do you do is a laser light is focused onto a fluorescent specimen through the objective lens. The mixture of reflected and emitted light is captured by the same objective and is sent to dichroic mirror. Now, reflected light is deviated by the mirror while the emitted fluorescent light passes through confocal aperture to reduce the out-of-focus light, and the focused light then passes through emission filter and processed to the photomultiplier. And in order to generate an entire image the single point is scanned in an XY manner as the laser focus is moved over the specimen.

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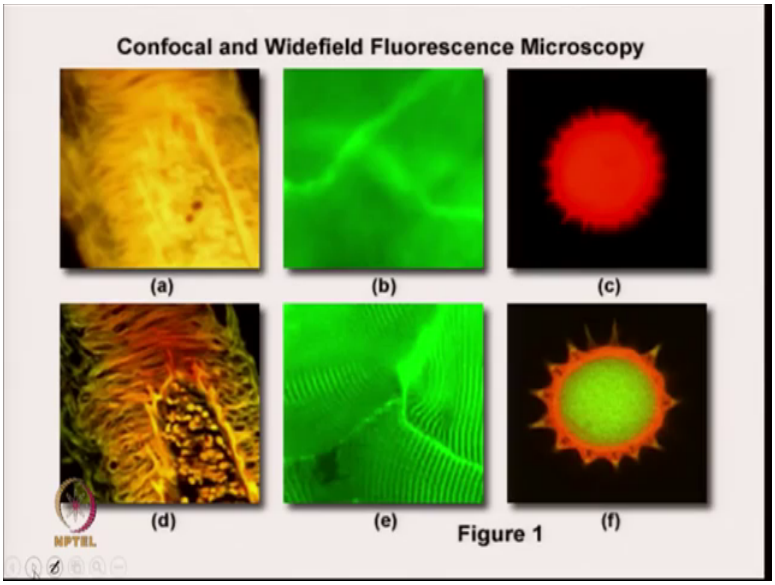

These are picture where comparison has been made between confocal and wide field microscopy, these are the picture obtained using wide field microscopy and these are the picture obtained using confocal microscopy. You can see that there is a quite better resolution in case of images obtained from confocal microscopy.

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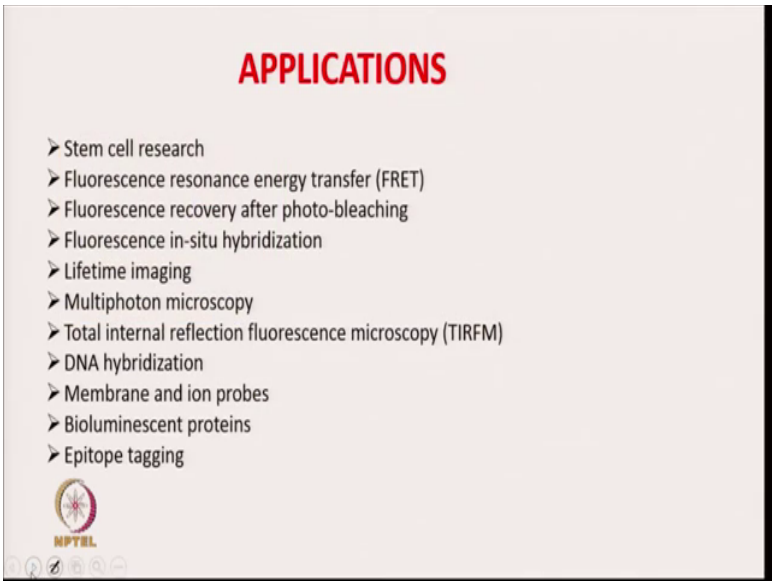

There are lot of application of confocal microscopy, in fact, all of the fluorescence microscopy, it has been utilized in stem-cell research. It is used to look at the interaction, applying the concept of FRET, also people look at fluorescence recovery after photo-bleaching and that I will discuss in the next lecture. Then in situ hybridization can be done. Lifetime imaging can be done, multiphoton aspect microscopy.

Total internal reflection fluorescence microscopy. DNA hybridization. Membrane and ion probe and bioluminescent protein and epitope tagging. There are several different kind of application is possible with confocal microscopy, I will discuss few of them today and then we will discuss it in the next class.

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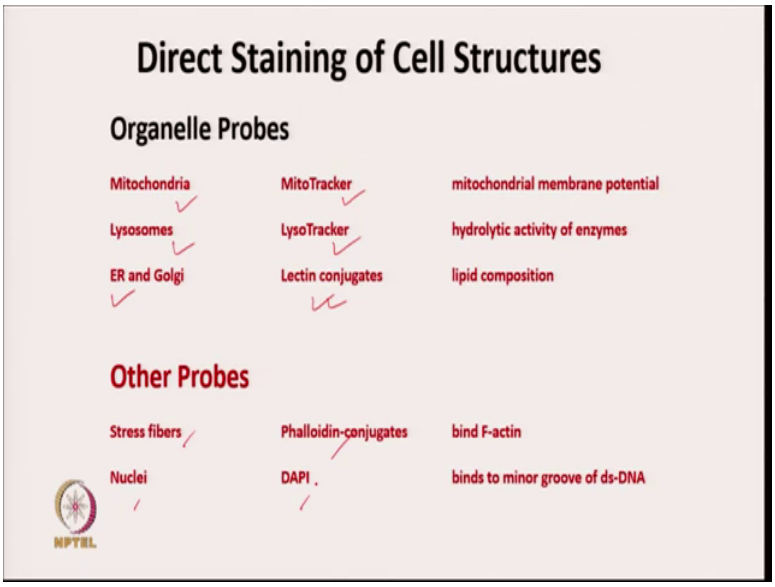

So, in fluorescence microscopy first thing is you do is, if suppose you want to look at the cell structure, first thing you do is, you directly stain the cell structures. For example, mitochondria, you use my MitoTracker, for lysosomes you have a LysoTracker and ER and Golgi you can use Lectin conjugates. For other probe, like stress fiber, Phalloidin-conjugates are used whereas to look at nuclei DAPI is used. So different part of the cells has different dyes, which can be probed by different dyes.

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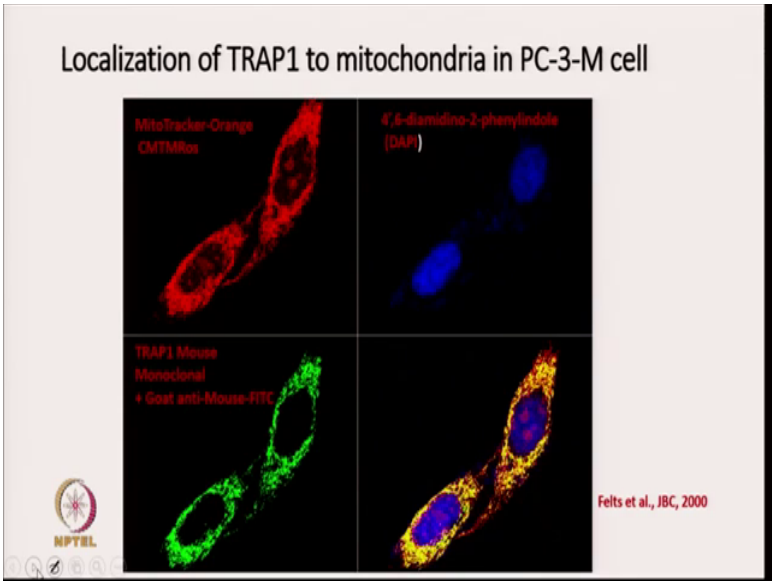

Now, let us look at this is the taken from a paper by Felts et al, JBC, 2000, where they looked at localization of Trap 1 to mitochondria in PC-3-M cells. And you can track mitochondria using situ tracker and from using DAPI you are looking at the nucleus, so this is your mitochondria and this is your nucleus. And if you look at trap 1 mass monoclonal plus, if you use antibodies corresponding to that are using FITC die what you will get in this picture. And then if you combine this you will get this picture and using this they were able to probe the localization of trap 1 to mitochondria in PC-3-M cell.

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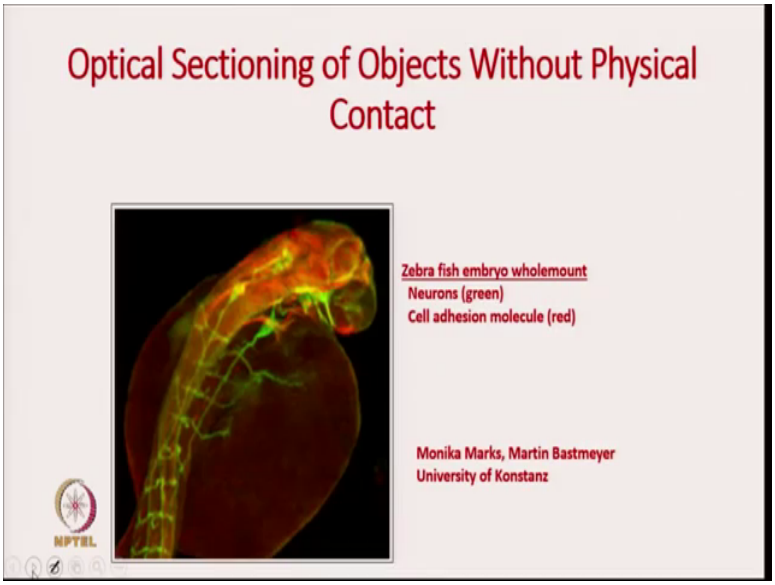

The confocal microscopy or fluorescence microscopy can be used for optical sectioning of objects without physical contact. What you need to do is, you just need to use the different

dyes. For example, here has been shown, a zebrafish embryo wholemount and neurons are shown in green, neurons are shown in green and cell adhesion molecule is shown red. So, since the different part of the specimen can interact with different dye and so they can be used to look at the optical sectioning of objects. We do not need to go look at the physical contact.

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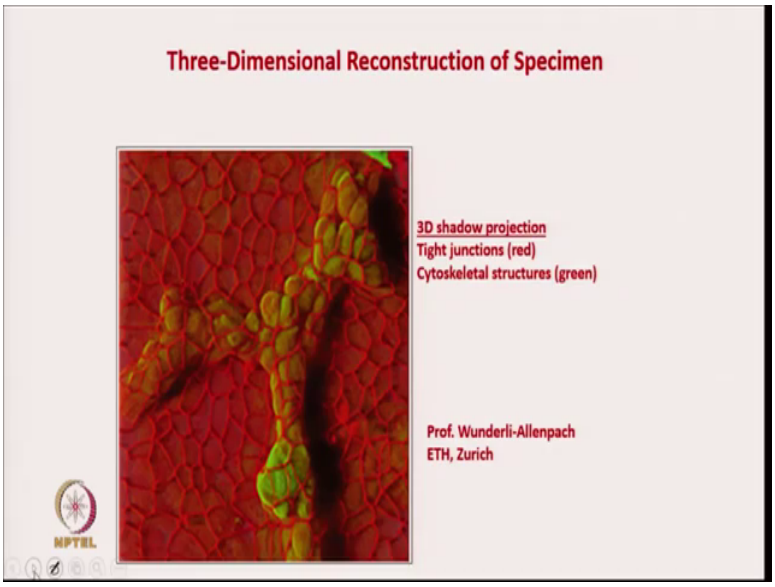

You can do 3-dimensional reconstruction of a specimen, for example, this picture is shown where you can see tight junction in the red, so these are the tight junction which can be seen in the red, and the cytoskeletal structure is seen in green, seen in green, so you can get a very beautiful 3-dimensional reconstruction of a specimen using fluorescence spectroscopy.

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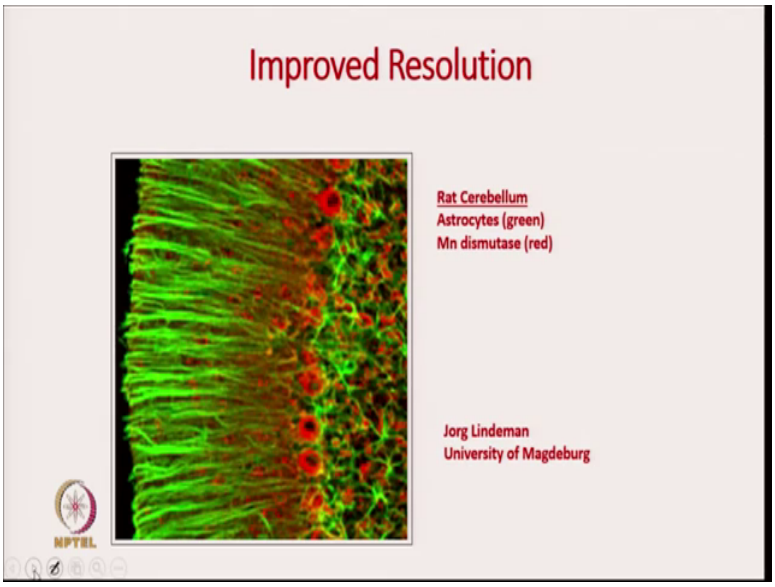

Now, you can see that what are good resolution you can obtain from a confocal microscopy. So, this is a picture of rat cerebellum and here Astrocytes are given in green and Min dismutase is shown as a red color. What is the distribution of astrocytes and min dismutase in the rat cerebellum has been shown here.

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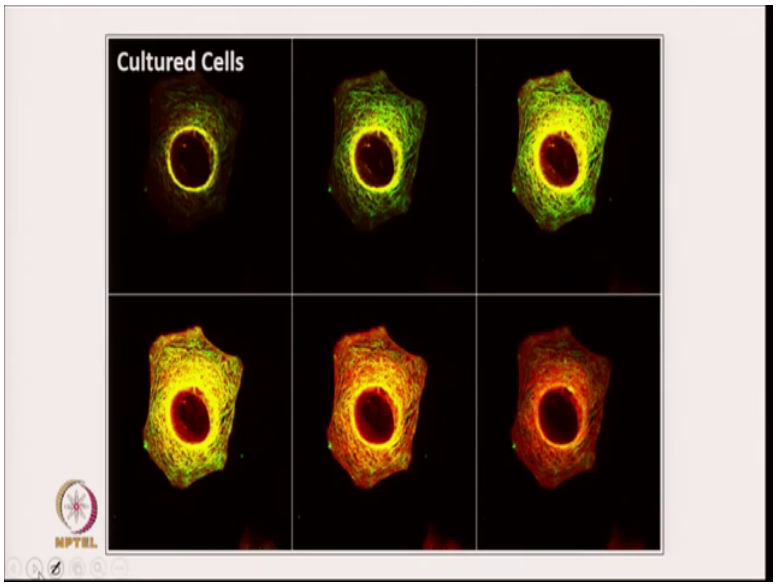

Even you can look at the culture cell and this is the cell growth at the time, the cell growth with the time so you can get a very nice picture of a cell morphology during a cell culture.

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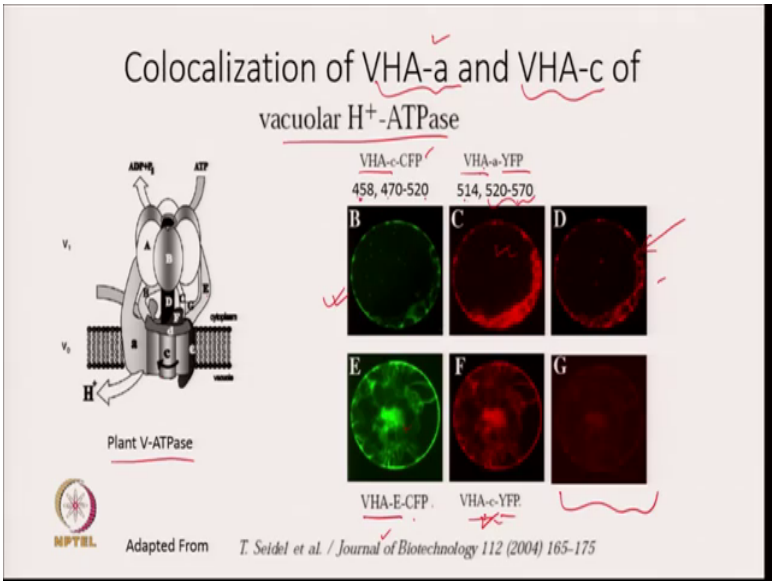

Now, we can also look at Colocalization of different proteins, so this is for vacuolar H plus ATPase. These are the two domain VHA-a if you see here, this is plant V-ATPase, this is plant V-ATPase and there are, you can see there is A domain, there is C domain, there is D

domain, there is E domain, so these are the different domains and if I want to know whether there is a co-localization of these two domain or not A and C. What can be done is, you see here, what has been done VHA-c has been tagged with CFP dye, Cyan fluorescence protein, and VHA-a is tagged with YFP dye.

Now, when you excite this at 458 nanometer and take an scan from 470-520 nanometer, you will get this spectrum, this kind of image. If you take this one and excite this to at 5, this at 514 nanometer and take a spectrum between 520 to 570, you will get this figure. And if suppose I excite this one at 458 nanometer or here at 458 nanometer and look a spectrum between 520 to 570 nanometer.

So, what I am doing is I am exciting at this 458 nanometer and I am looking spectra as 520 to 570 nanometer, you can see that still I am able to see this beautiful image, this beautiful image. When that can be possible if 2 these domains are close enough, so that FRET can take place, FRET can take place and that is what is happening here and what it does tells you, that VHA-c and VHA-a are co localized.

Now, let us take the two different domain here VHA-E and VHA-C. So, now, what we are looking at co-localization of E with C. Again it has been tagged with CFP and this is tagged with YFP and you excite at 458 and look between 470 to 520, will see a beautiful picture. You excited 514 nanometer this one and look at this spectra between 520 to 570, there is a beautiful picture.

But now if I try to do this from excited 458 nanometer and look the spectra at 520 to 570, you see me, do not see good illuminated picture, what does that mean is, what does this mean is that VHA this E, capital E and VHA-C domain are not co-localized, they are not co-localized.

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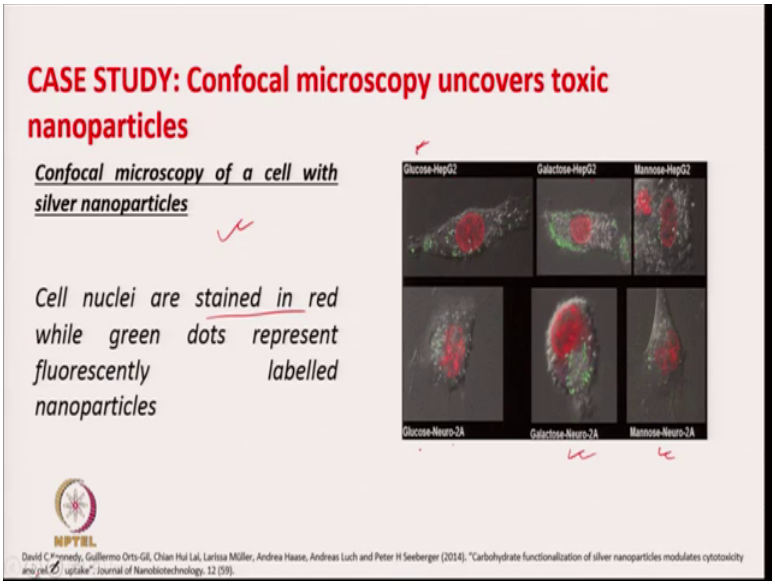

Then it can be confocal microscopy can uncover toxic nanomaterials and here it has been shown microscopy of a cell with silver nanoparticle, cell nuclei are stained in red, while green dots represents labelled nanoparticle. So, you can look at, so here you see glucose with the HepG2 cells, galactose HepG2 cells, mannose HepG2 cells, and glucose Neuro2A cells, this is at the Neuro2A cells and now you can see that it is of glucose nano, so glucose.

So, what you can see is different distributions of this nanoparticles, different distribution of these nanoparticles, and you can tell that these nanoparticles are distributed differently and these cells.

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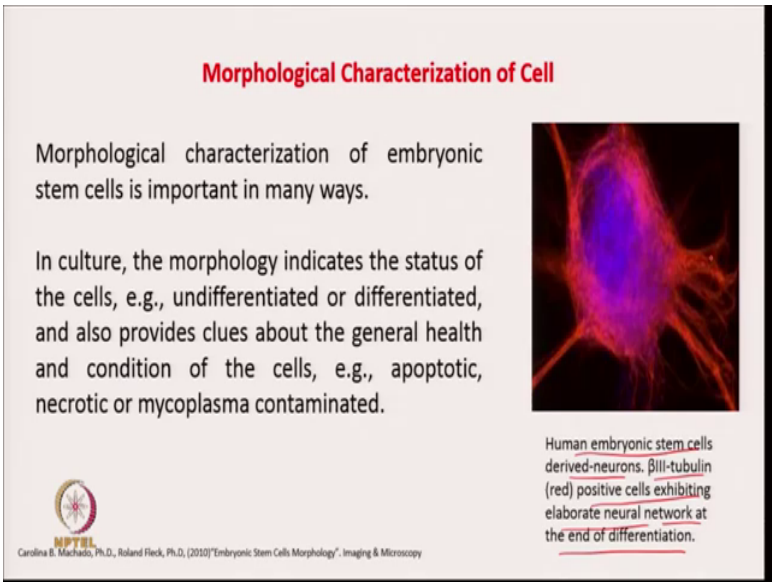

You can also do morphological characterization of a cell. Morphological characterization of embryonic stem cell is important in many ways. So, in culture, morphology indicates that status of the cell, example, undifferentiated or differentiated, and also provide clues about the general health and condition of the cell, for example, whether the cells are apoptotic, necrotic or mycoplasma contaminated.

And the morphology of the cells can be seen, morphology of the cells can be seen through confocal microscopy, so this is human embryonic stem cells, there are neurons. Beta 3 tubulin which is the red positive cells exhibiting elaborate neural network at the end of the differentiation. So, if you are at the end of differentiation then you will see elaborate neural network otherwise you will not see and thus, we can tell you about other cells are undifferentiated or differentiated whether cells are in apoptotic condition or necrotic condition or mycoplasma contaminated.

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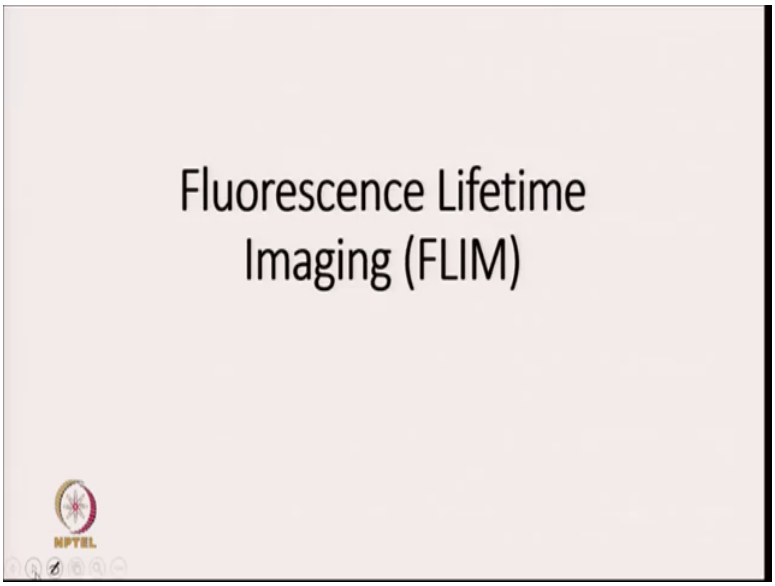

The next technique is fluorescence lifetime imaging flim and as the name suggests this is based on a lifetime measurement, based on lifetime measurement which we have already discussed. Now this, that lifetime fluorescence is now being seen through your microscopy.

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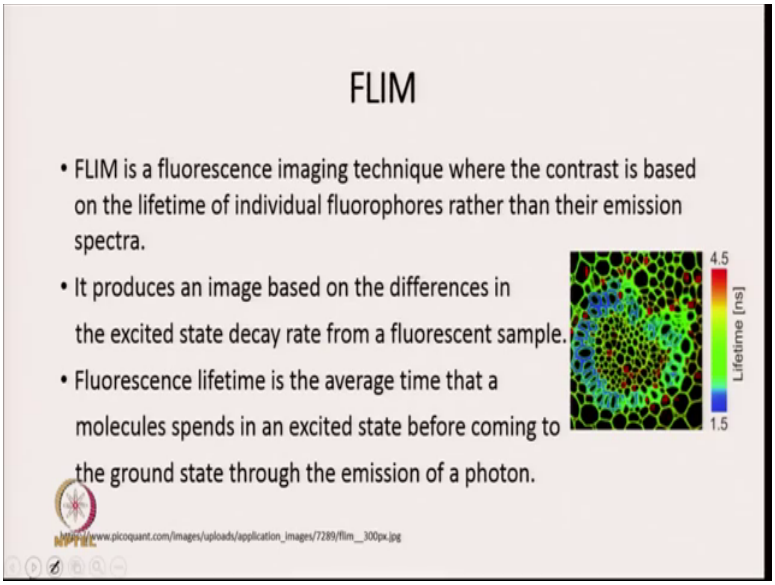

So, flim is a fluorescence imaging technique where the contrast based on the lifetime of individual fluorophores rather than their emission spectra. It produces an image based on the differences in excited state decay rate, from decay rate from a fluorescence sample. And fluorescence lifetime is the average time that a molecules spends in excited rested before coming to ground state through the emission of photon, that we have already discussed several time. So, now you can see this is your image and you see red as higher lifetime, blue has lowest life time, so basically you can see the picture of a specimen quite clearly based on the lifetime.

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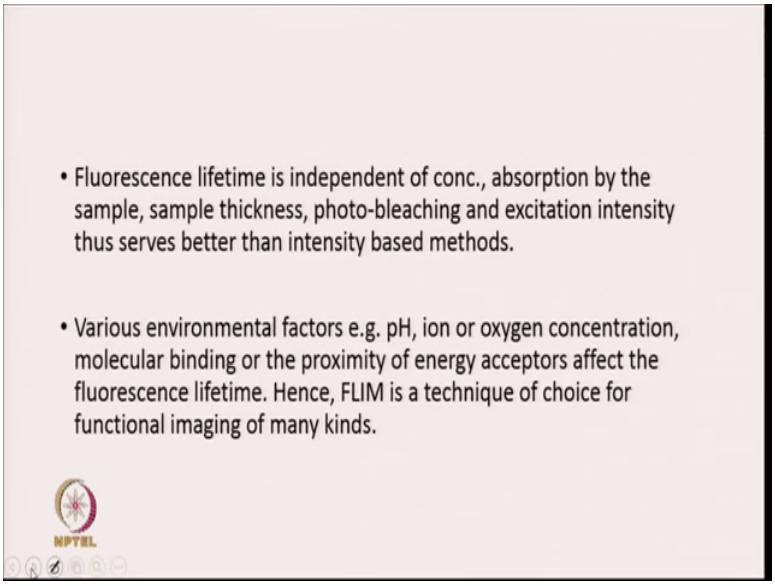

One of the very important feature of fluorescence lifetime is that it is independent of concentration, absorption by the sample, sample thickness, photo bleaching and excitation intensity and thus, it serves better than intensity based methods. But life time is affected by environmental factors, for example, pH, ion, oxygen concentration or molecular binding or proximity of energy acceptors and thus, if we want to look at the functional imaging it is a much better choice.

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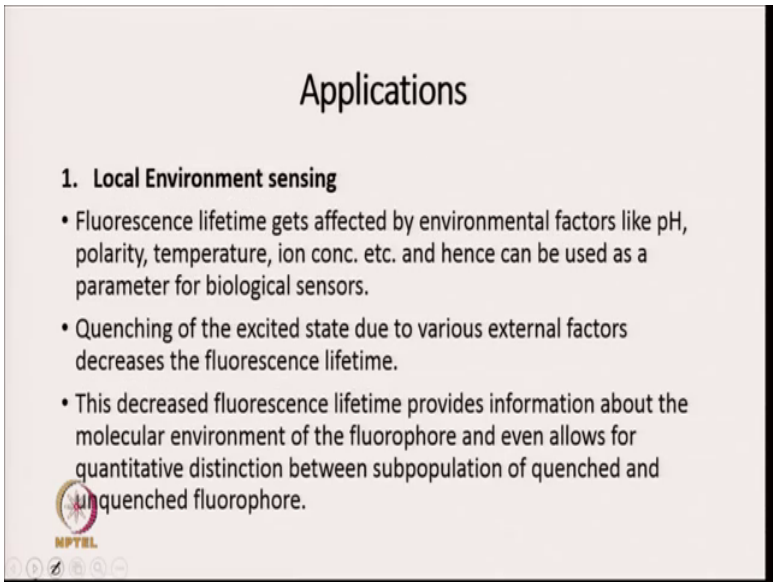

For example, let us look at the local environment sensing, I already told you that florescence lifetime gets affected by environmental factors and so it can be used as a parameter for biological sensors. Quenching of excited state due to various external factors decreases the lifetime. And this decreased fluorescence lifetime provide information about the molecular environment of the flows and thus, also allows quantitative distinction between subpopulation of quenched or unquenched fluorophore.

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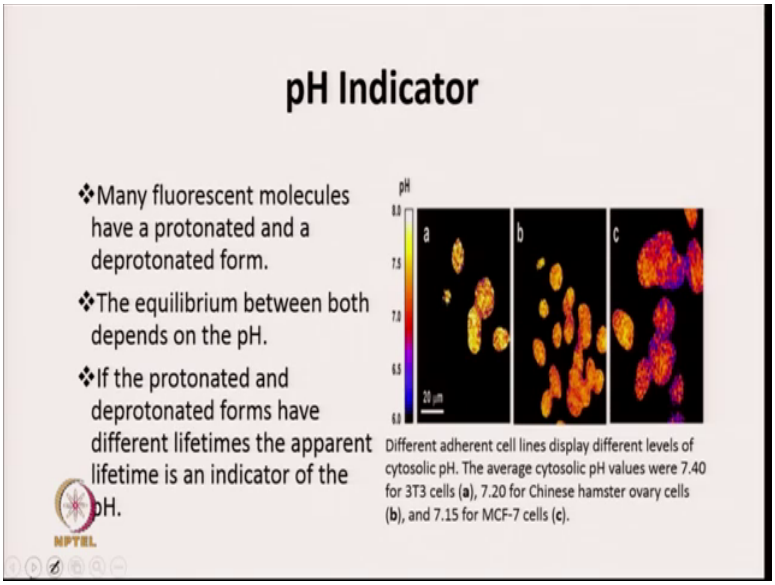

So, this flim can also be, can be utilized as flim can be utilized that pH indicator. Many fluorescent molecules have protonated and deprotonated form, so there is equilibrium exists between two forms depending on pH and if protonated and deprotonated form a different lifetime, the print lifetime is an indicator of pH. Apparent lifetime can be used as an indicator of the pH.

And here it has been shown, different adherents cell line display different level of cytosolic pH. The average cytosolic pH values are 7.40 for 3T3 cells, 7.2 for Chinese hamster ovary cells and 7.15 for MCF-7 cells, MCF-7 cells. So, you can use an indicator or dye which exists in both protonated and deprotonated form and their equilibria changes with the pH and that is the way you can differentiate or you can use them, that way you can use them to differentiate the pH of a particular place in our body.

So pH of different cell has been examined using these dyes and some of the examples are given here. The first one is for, this one is for 3T3 cell, the second one for Chinese hamster ovary cells and the third one is for MCF-7 cells and the different pH value which is the pH value obtained for the cells are different.

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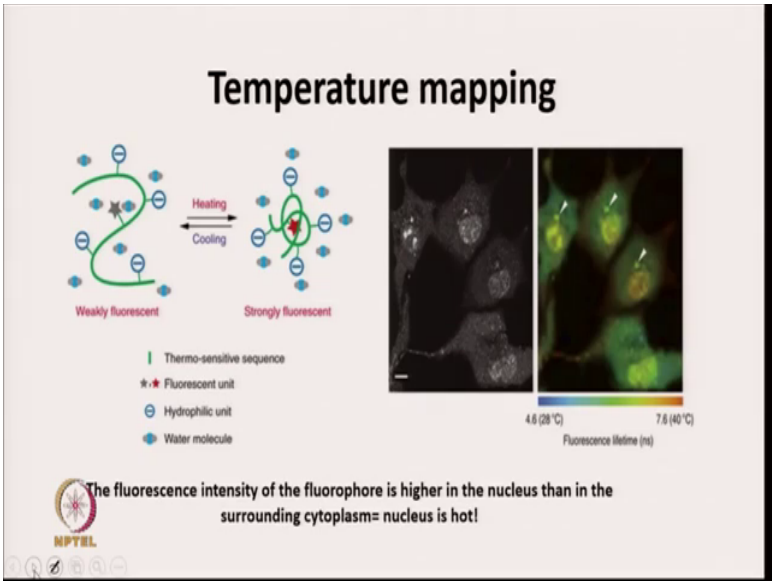

We can also map the temperature inside a cell for that we use the fluorophores which are sensitive to temperature. So some of the fluorophores are weakly fluorescent when it is at a lower temperature whereas they are strongly fluorescence when they are at higher temperature and so they can be used to look at the temperature. Now, you see here this is the same cells and this is 4.6 28 degree Celsius 7.6 440 degrees Celsius this is the different color.

So in a cell you can see that there are different color inside the cell and that is because of different temperature at the different point. So fluorescence intensity of fluorophore is higher in the nucleus and that is why you see this kind of color. Then in surrounding cytoplasm, what does that mean is nucleus is hot. So, temperature mapping inside the cell can be done using the flim.

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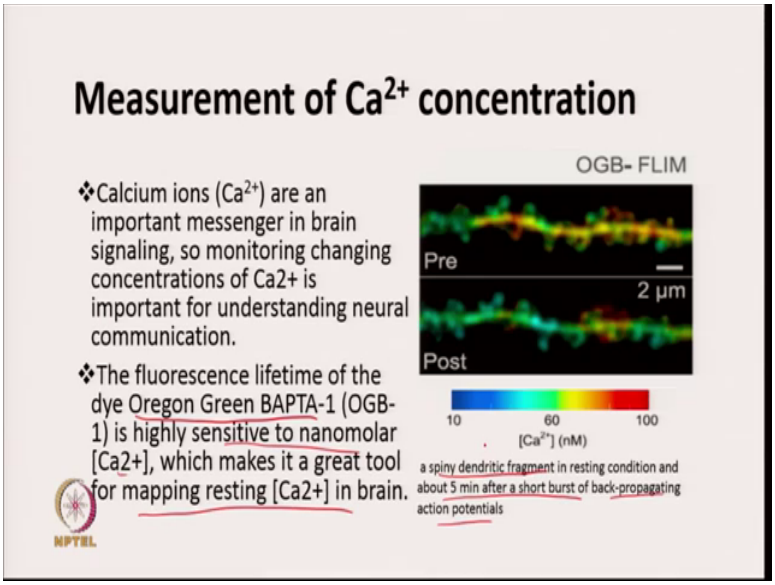

We can also measure the calcium 2 plus concentration, calcium ion are an important messenger in brain signaling, so monitoring changing concentration is important for understanding neural communication, for that the dye is used is a Oregon green BAPTA-1, and it is highly sensitive to nanomolar calcium 2 plus concentration and which makes it a great tool for mapping resting calcium 2 plus in the cell.

And now you can see here it is a spiny dendritic fragment in resting condition and about five minutes after short bursts of back propagating action potential color change and this color is depending on the your calcium 2 plus concentration, calcium 2 plus concentration.

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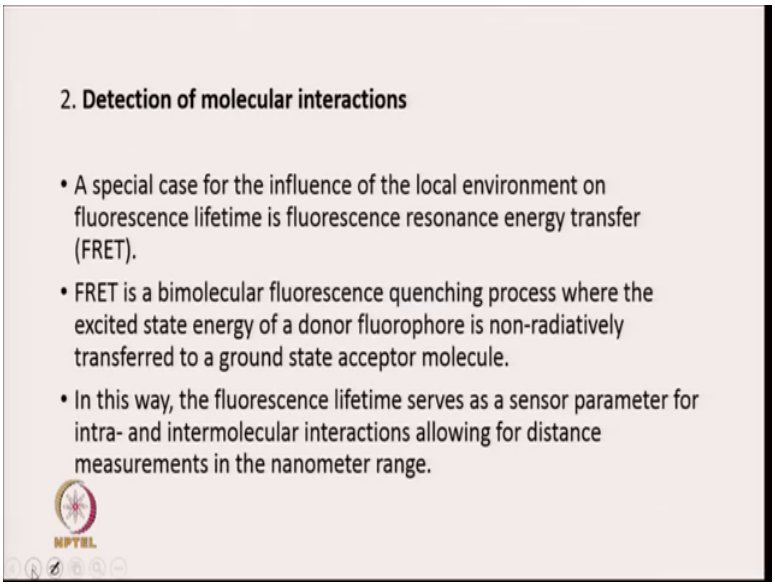

We can also use flim to detect the molecular interactions and a special case for the influence of the local environment on the fluorescence lifetime, is known as FRET we have already discussed it. FRET is a by molecular fluorescence quenching process where the excited state of a donor fluorophore in non-radiative transferred to a ground state acceptor molecule. In this way, fluorescence lifetime serves as a sensor parameter for intra and intermolecular interaction allowing for distance measurement in nanometer range. So, FRET changes the lifetime and so flim can be used to look at the interaction between two different molecules.

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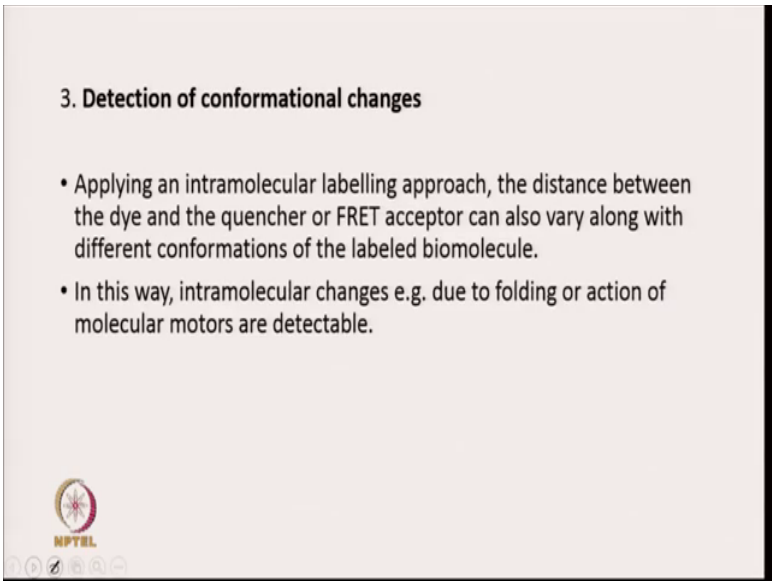

We can also look at conformational changes, so applying an intramolecular labelling approach, the distance between a dye and quencher of FRET acceptor can also vary along with different conformation of labelled biomolecules. So, in different conformation of biomolecule the distance between dye acceptor will change and hence just by looking at the distance, we can tell what is the changes happening when you go from one conformation to other. So, in this way you can look at intramolecular changes due to folding or action of various motors.

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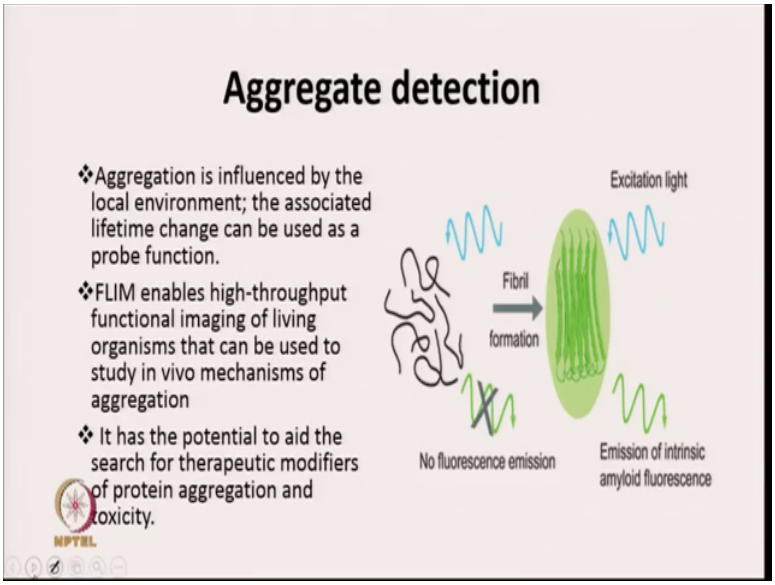

We can also detect aggregation, aggregation is influenced by local environment; the associated lifetime changes can be used as a probe function, flame enables high-throughput functional imaging of living organism, that can be used to study in vivo mechanism of aggregation. It has the potential to add the search for therapeutic modifiers of protein aggregation and toxicity.

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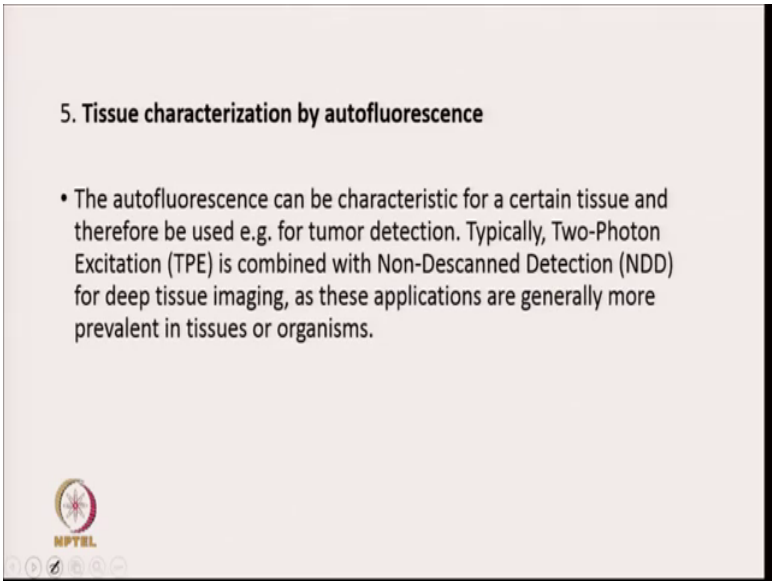

Tissue can also be characterized using by using simple auto fluorescence, fluorescence from intrinsic fluorophore, the auto fluorescence can be characteristic for certain tissues and therefore, can be used for tumor detection and there are several important methods which can be utilized for that.

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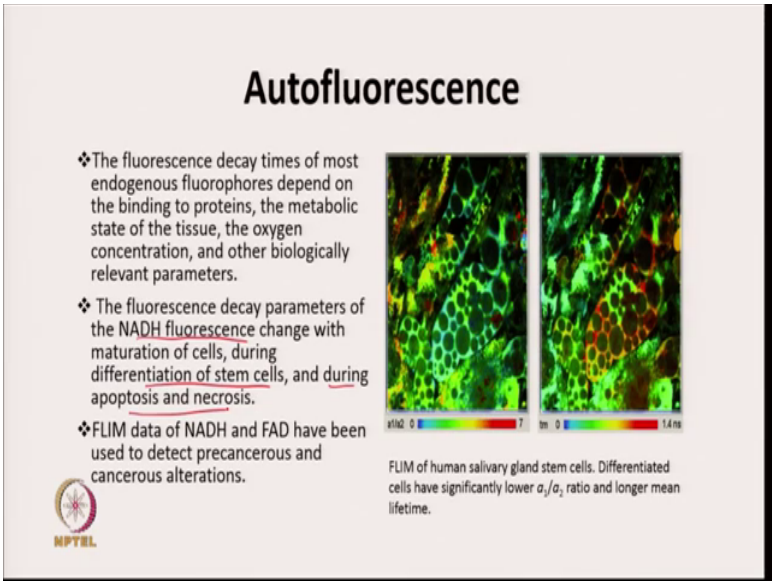

The fluorescence decay time of the most endogenous fluorophore depend on binding to protein, the metabolic state of tissues, the oxygen concentration and other biologically relevant parameter. The fluorescence decay parameter of NADH fluorescence changes with maturation of cells and during differentiation of stem cells and during apoptosis and necrosis. So, flim data of NADH and FAD has been used to detect precancerous and cancerous alteration.

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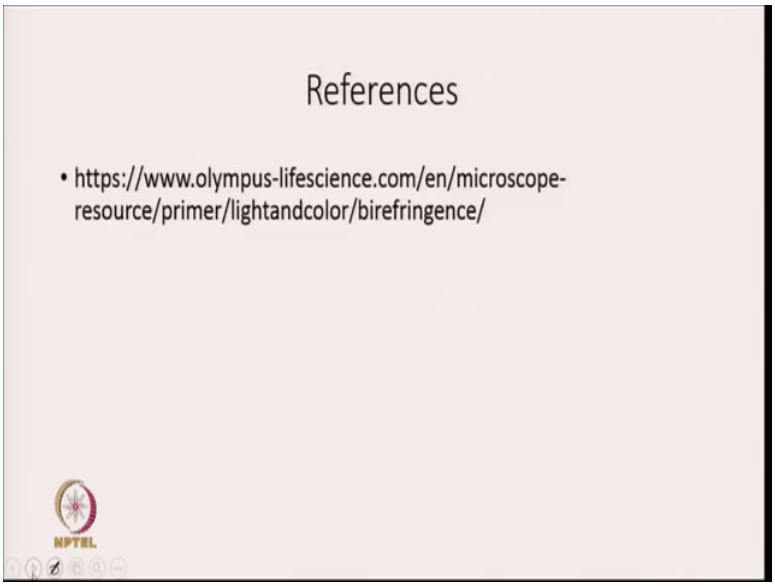

So, there are a lot of applications of this confocal fluorescence and flim. Some of them I have discussed today, the others we will discuss in the next lecture, thank you, bye.