Spectroscopy Techniques for Pharmaceutical & Biopharmaceutical Industries
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Lecture 29
Fluorescence Microscopy & Application

Hello students, welcome back to lecture 29 of this course, last time I started discussing about fluorescence microscopy, I will continue with that and show you some more applications. Fluorescence Microscopy has several advantages as we know that fluorophores are self-luminous, so they exhibit very high contrast, they are also very sensitive we only require small concentration for successful labelling of the different components.

They are very specific and so they can discriminate between individual proteins, they can discriminate between organelles and they can discriminate between components of cells very well. Another advantage with fluorophores are that they can be tolerated by living cells in low concentrations.

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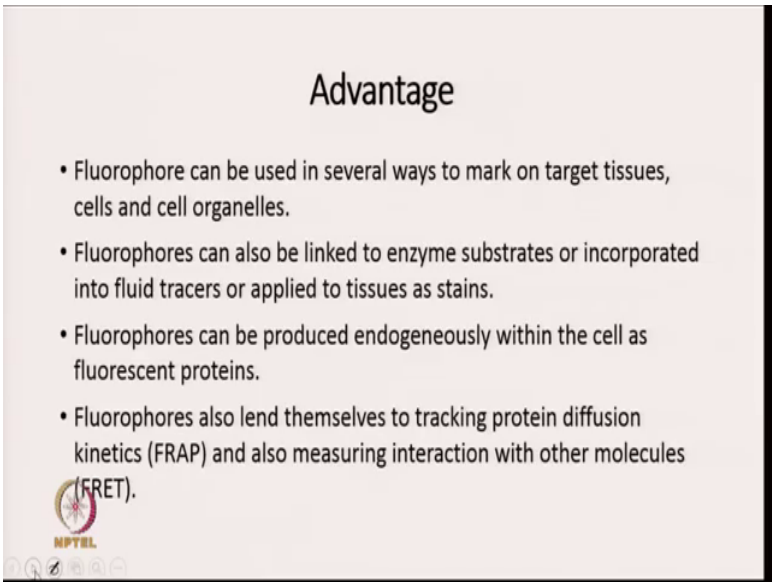

Fluorophore can be used in several ways to mark on target tissues, cells and cell organelles. And so if you want to look at cell and its component, fluorescence microscopy is very useful. The fluorophore can be used in several ways to mark on target tissues, cells and cell organelles and that is why fluorescence spectroscopy is quite often used to look at cell and cell organelles.

Fluorophore can also be linked to enzyme substrates or incorporated into fluid tracers or applied to tissues as stains. They can be produced endogenously within the cell as

fluorescence protein. And they can lend themselves to tracking protein diffusion kinetics and also for measuring interaction with other molecules. So there are several different applications of fluorescence as spectroscopy and I will show you some examples to you.

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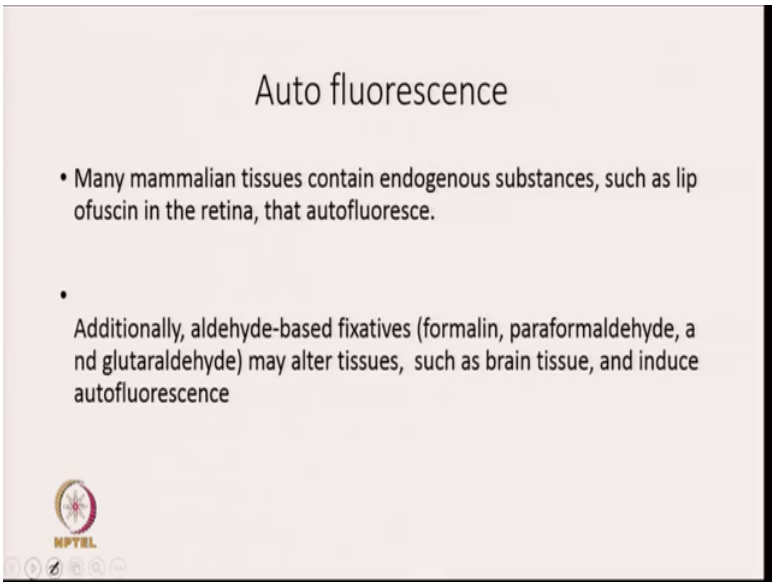

So some cell tissues, so auto fluorescence and that is how you can differentiate between two or those such tissues. So many mammalians tissues contain endogenous substances, such as lipofuscin in retina, that auto fluoresce. And there are few tissues which induce auto fluorescence on treatment with aldehyde based fixatives. And thus you can look at those tissues and see what is happening to that tissue.

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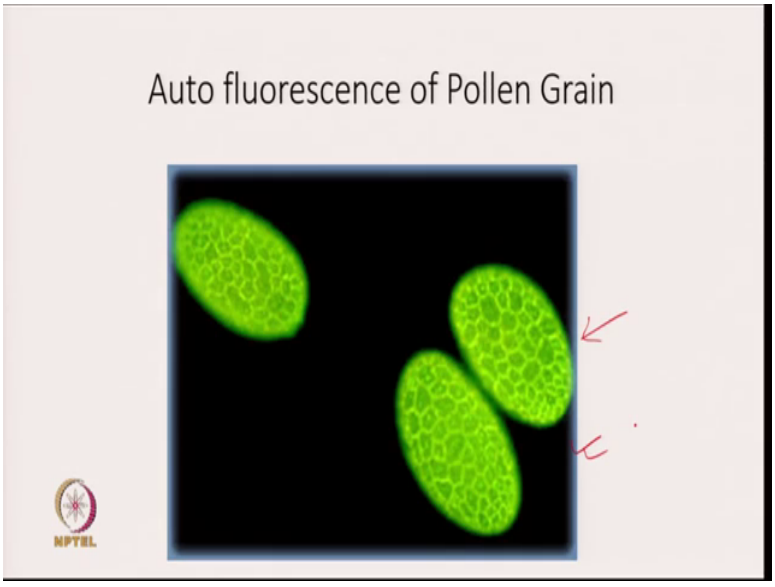

This is a image which shows you auto fluorescence of pollen grain, so this has been taken from fluorescence microscopy and you can see that you can see auto fluorescence of pollen grains.

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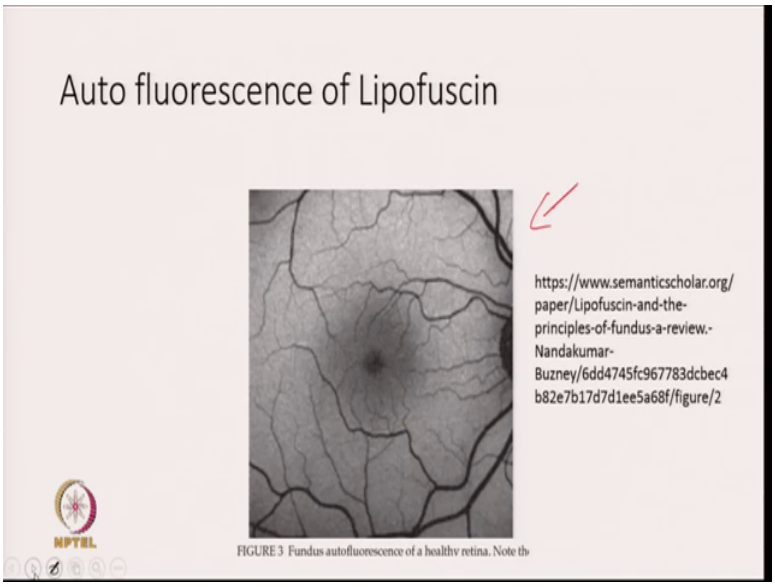

Lipofuscin of retina also has auto fluorescence as I told you and that can be seen from this fluorescence microscopy image and you can see everything is quite clear here. So dark lines can be seen, so just from auto fluorescence you can see the different tissues.

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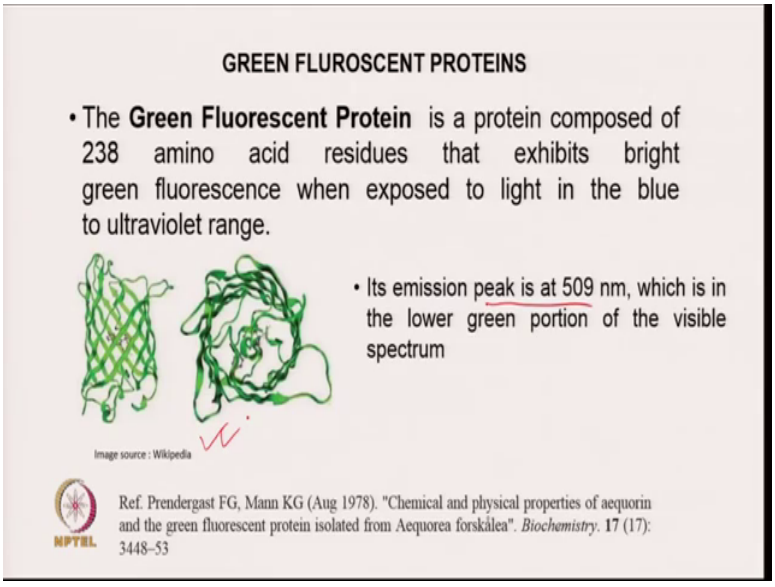

Some of the tissues can be seen with auto fluorescence. But some of the applications need the use of what is known as green fluorescent proteins. So let me discuss first green fluorescent

proteins and then I will discuss how they can be used for various applications. Green fluorescent protein is a protein which has 238 amino acid residues and they exhibit bright green fluorescence when exposed to light in blue to ultraviolet range.

And its emission peak is at 509 nanometre, which is in the lower green portion of the visible spectrum and that is why they looks like green. And you can see the green fluorescent protein picture taken from two different angles.

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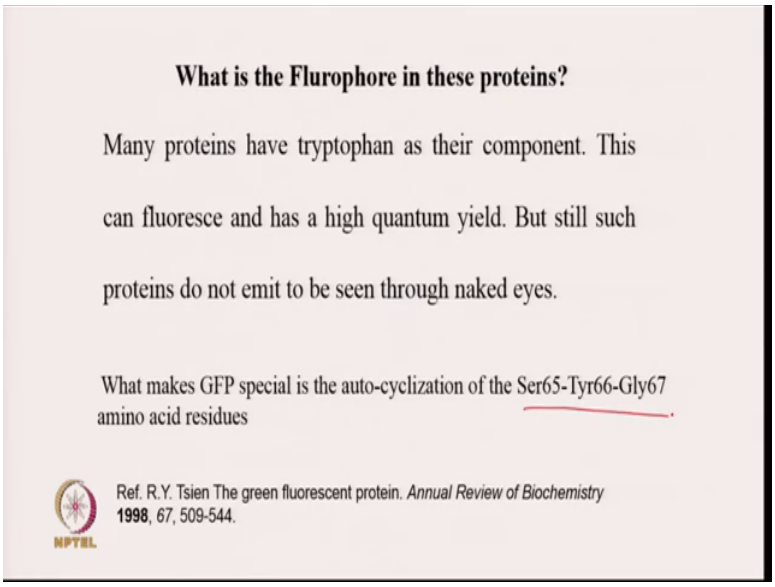

Although many proteins have tryptophan as their component. Which can fluoresce and has a high quantum yield. But they do not emit the fluorescence quite enough to see through naked eyes. So what makes GFP Green Fluorescent Protein special is the auto-cyclization of these three residues serine 65, tyrosine 66, and glycine 67. So GFP has this tri amino sequence which undergoes auto-cyclization in presence of light and that makes it fluoresce more.

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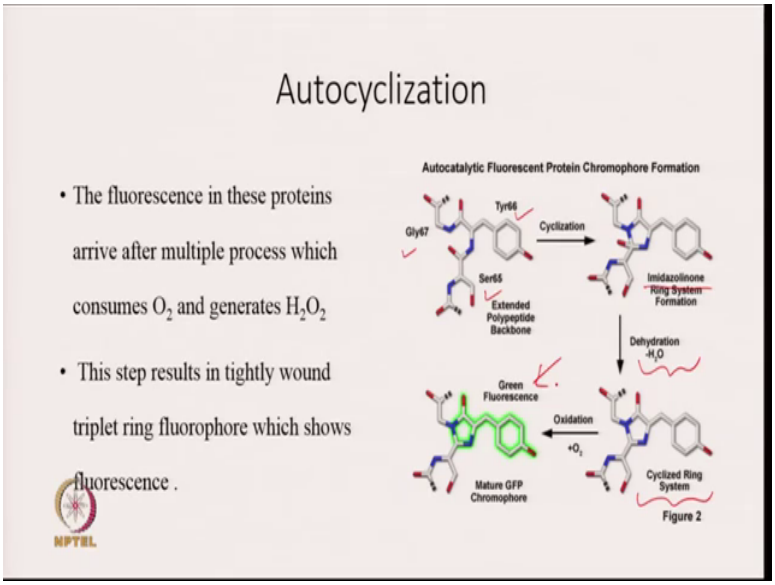

So what happens basically is you can see the serine 65, tyrosine 66, and glycine 67. It undergoes cyclization to form imidazolinone ring system and it undergoes dehydration to give you cyclized ring system, cyclized ring system, which on oxidation, which on oxidation gives you green fluorescence and this is known as mature GFP chromophore. So the fluorescence in GFP arrive after multiple process which consumes oxygen and generates H 2 O 2 and this result into tightly wound triplet ring fluorophore which shows fluorescence.

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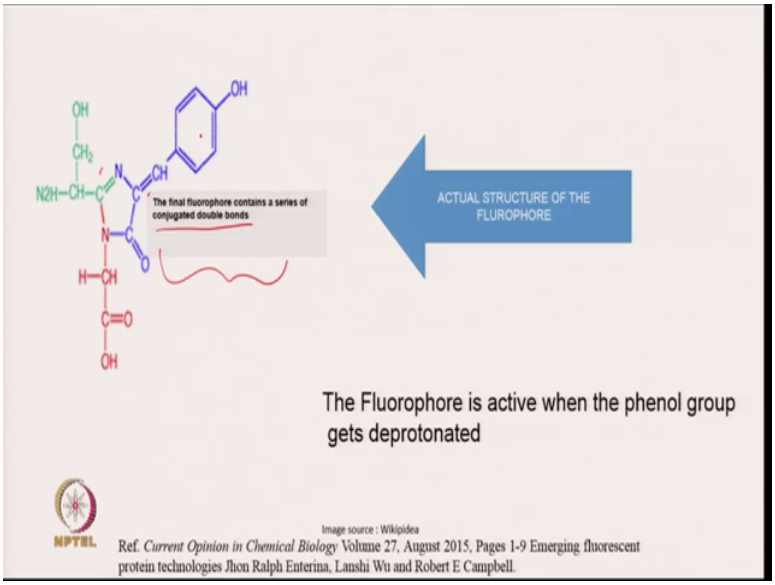

And this is the final structure of your fluorophore, cyclized fluorophore. It has series of conjugated double bonds and you can see that this is conjugated to this double bond and this is conjugated to this double bond. And so there is a series of conjugation and that is why it

fluoresces. The fluorophore is active when the phenol group gets deprotonated. So if it gets deprotonated then there is a conjugation and then it gets active, it gets active.

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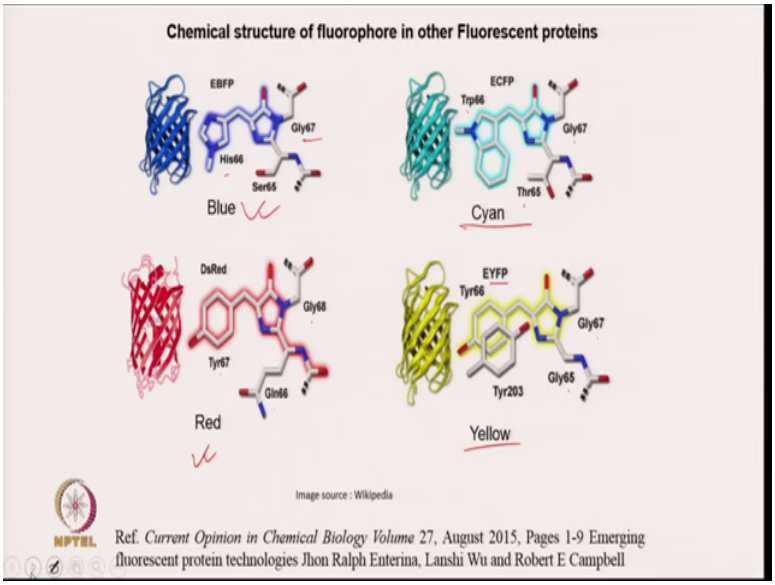

Here are some more fluorophores which is protein based and their structures are given, this is the blue fluorescence protein it is known as EBFP, and this as cyan and this is known as ECFP so cyan fluorescence protein, this is blue fluorescence protein. And then you have a red colour this is known as Ds Red and this is yellow fluorescence protein which has yellow colour. And this has somewhat different colours, this is glycine 67, histidine 66, serine 65.

If you see here threonine 65, glycine 67 and tryptophan 66 gives you CFT. Glutamine 66, tyrosine 67 and glycine 68 gives you Ds Red. And glycine 65, tyrosine 66 and glycine 67 gives you YFP. So these are the fluorescent proteins which I endogenously expressed as a fusion protein and then you can track the different proteins or nuclei using this.

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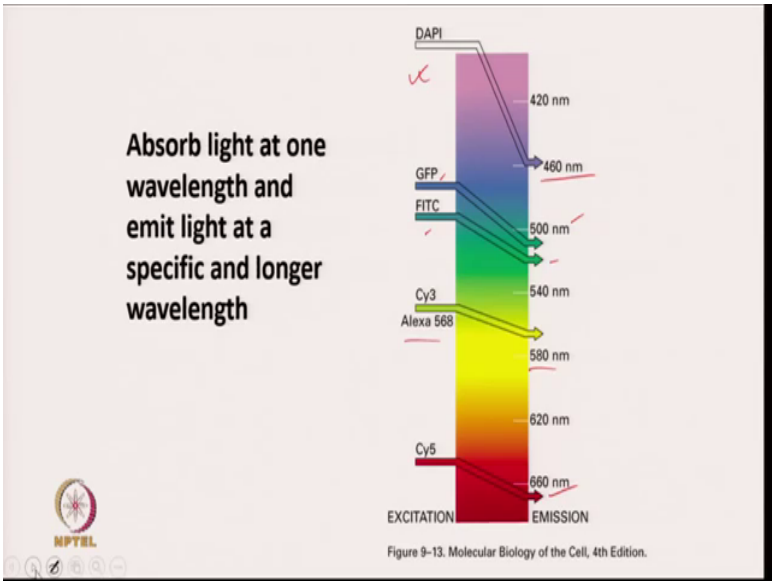

So these are the few fluorophores which are quite often used DAPI which I discussed last time it emit at 460 nanometre. GFP green fluorescent protein it emits at 500 nanometre. FITC its emits at around 520 nanometre. Cy3 or Alexa 568 it emits at 580 nanometre. And Cy5 it emits at 660 nanometre.

So this is they absorb at one wavelength and emit light at specific and longer wavelength, so you can see that this side if you go this is a lower wavelength so they are absorbing at lower wavelength and emitting at high wavelength and that is known as (())(10:29) that we have already discussed.

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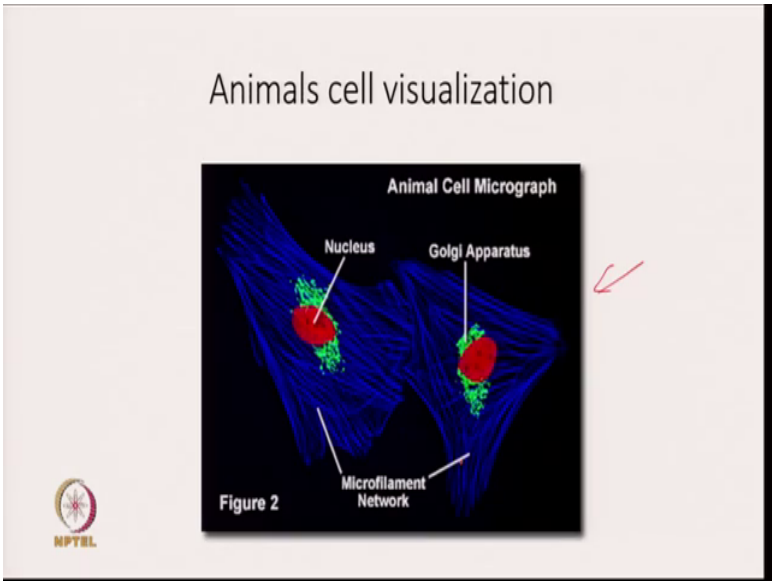

Now there are several application which I discussed with you last time, but I will also show you more applications of fluorescence microscopy. We can use fluorescence microscopy for animal cell visualization, here is one animal cell micrograph. You see this is your Golgi apparatus, this is nucleus and then microfilament network they can be stand with the different eyes and you can see it clearly in a cell, you can see it clearly in an animal cell and that is what is the beauty of fluorescence microscopy, you can see clearly all these organelles inside an animal cell.

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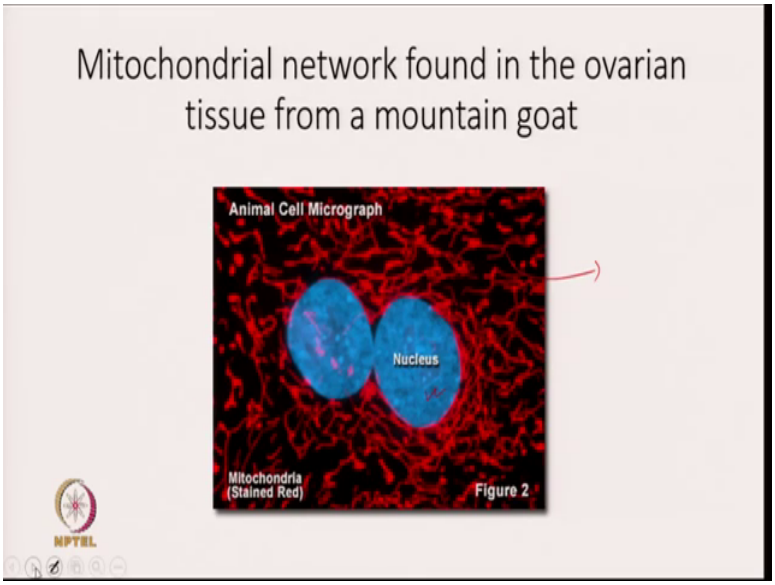

Now this is another image taken from fluorescence spectroscopy. And in this mitochondrial network was found in ovarian tissue from mountain goat, so these all red one is mitochondrial network and this is the blue one is nucleus, we can clearly see how mitochondrial network is there in ovarian tissue from mountain goat.

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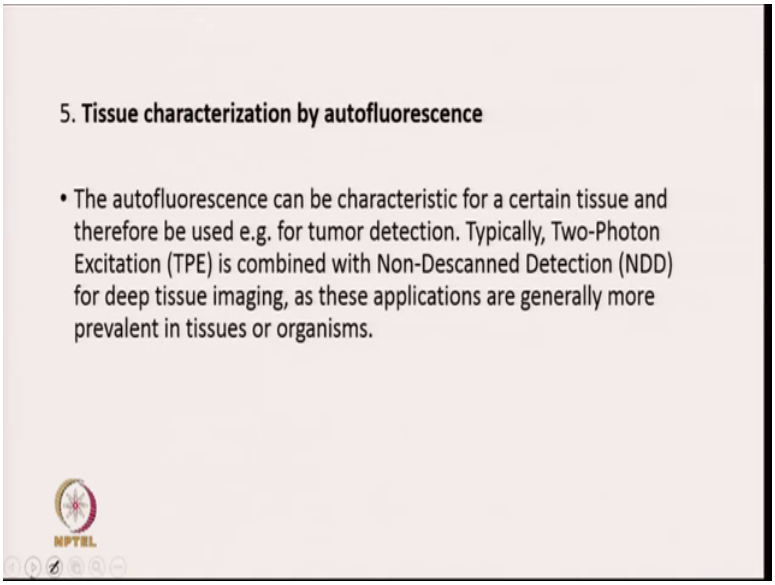

We already, I already discussed about auto fluorescence. Auto fluorescence can be characteristic for a certain tissue and therefore can be used for tumour detection also.

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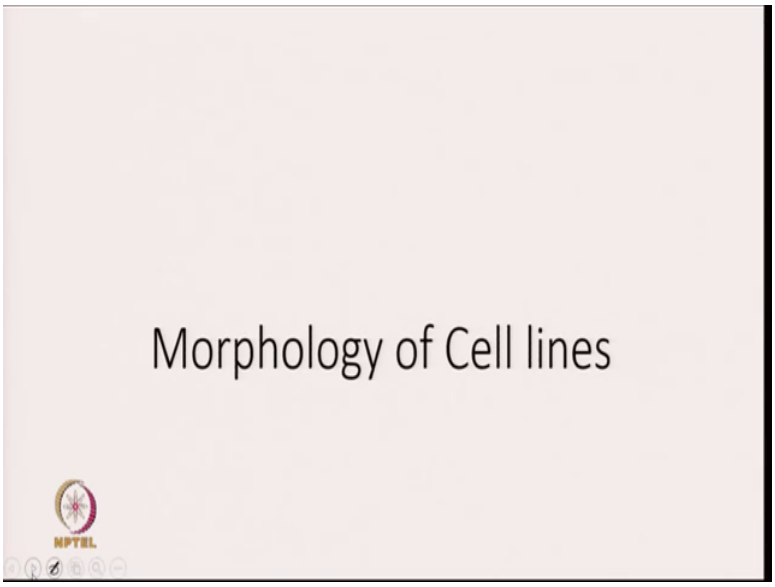

Not only we can look at the different parts of a cell, we can also see morphology of cell lines.

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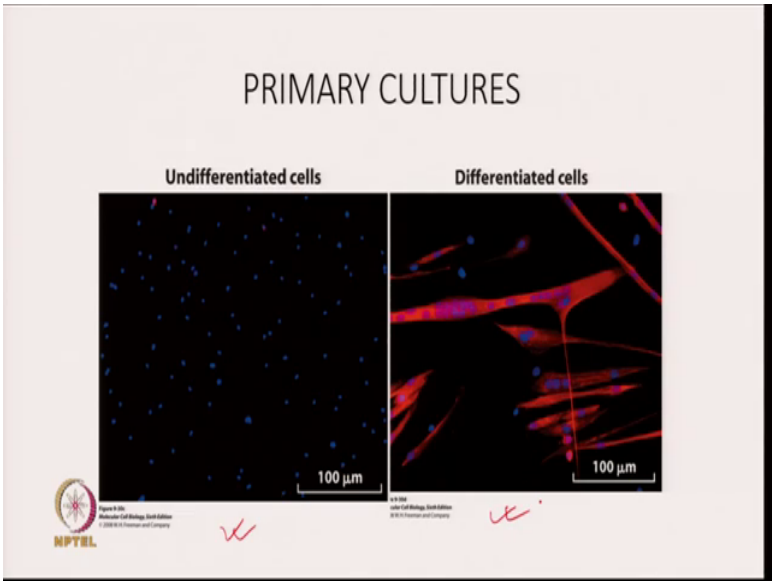

And here is one example given. This is an example of undifferentiated cells, so you can see how does it look like in a fluorescence microscope. And now when you take a differentiated cells how will this look like. So you can differentiate between different types of cells or different cells in different conditions, cells in different status. So one example here is given of undifferentiated cells, and this is given for differentiated cells. There is remarkable difference between images of two different cells.

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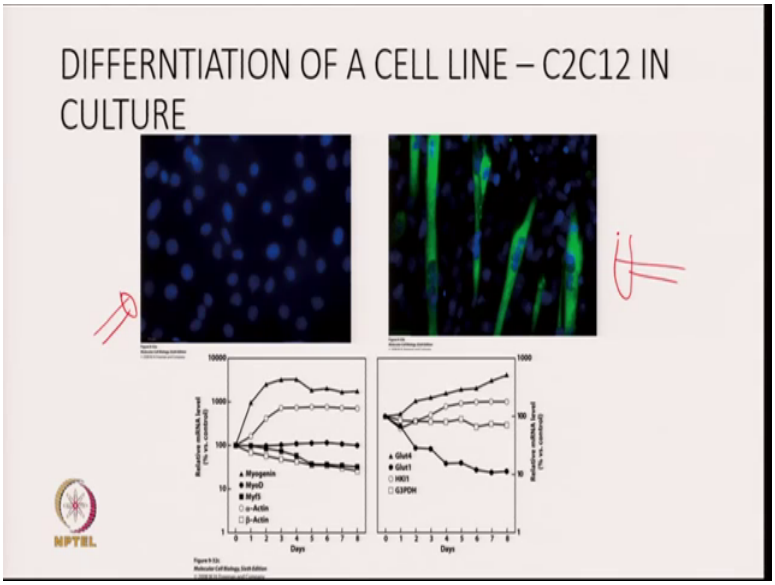

This is another example, where we looked at, where researchers looked at differentiation of a cell line C2C12 in culture. And that you can see the initial picture is like this and then differentiated cells looks like this, differentiated cells looks like this.

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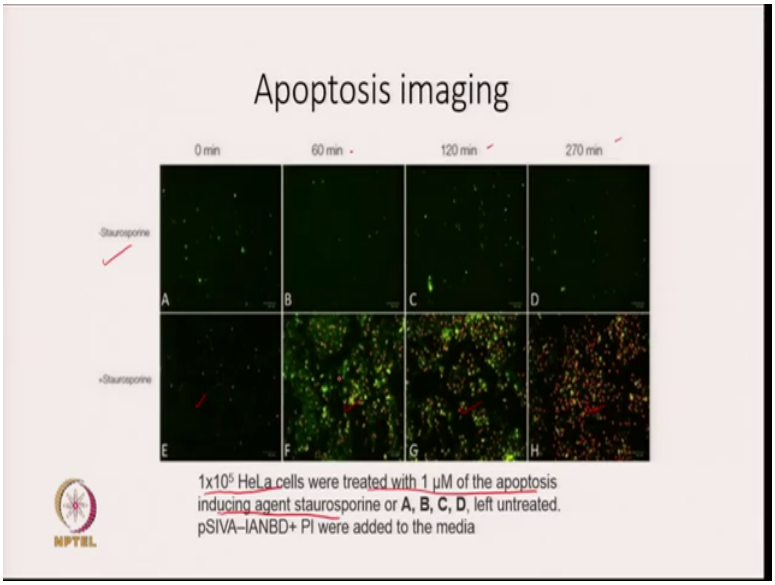

We can also image the phenomena of apoptosis using imaging, fluorescence imaging. Here is the example where 1 into 10 to the power 5 HeLa cells were treated with 1 micro molar of apoptosis inducing agent staurosporine and you can see this is without staurosporine at 0 minute, 60 minute, 120 minute, 270 minutes and you can see there is very little difference. But when you add this reagent which is basically apoptosis inducing agent, this is at 0 minute and then 60 minute, 120 minute, 270 minute.

So you can see there is a vast difference when you go from 0 minute to 270 minute, you can see all this spots brighter spots quite clearly in apoptotic cells and their colour is also changing, their colour is also changing. So not only, so microscopy, fluorescence microscopy cannot only be used to look at that organelles of different cells, we can also see the status of cells.

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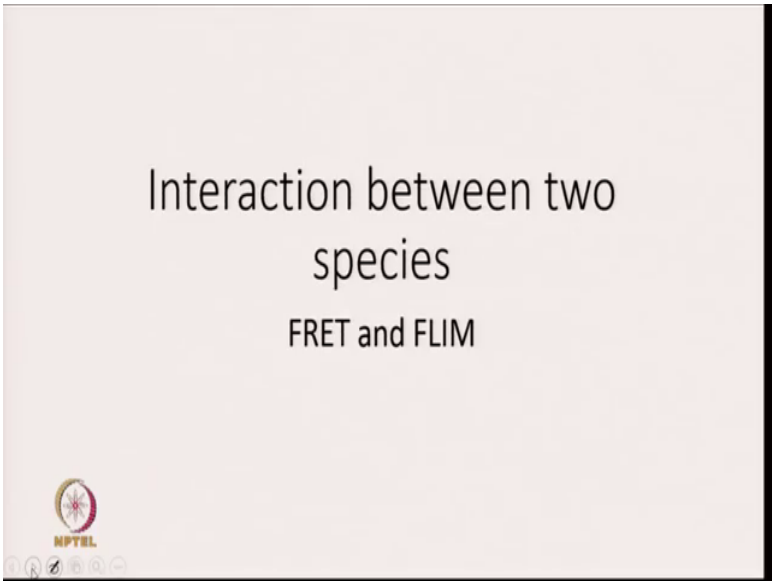

Fluorescence microscopy can also be used to study interaction between two species and for this we use the special technique of fluorescence FRET and FLIM.

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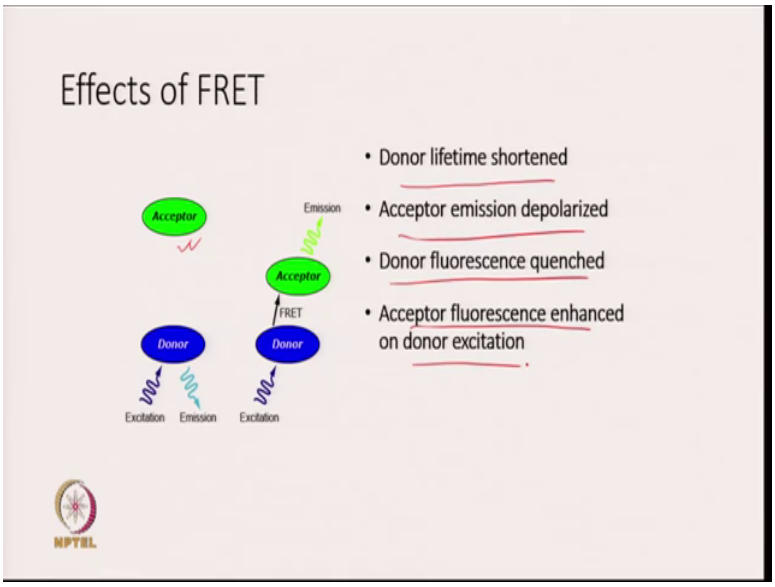

I have already discussed FRET and FLIM, but let me again give you a brief overview. So, when a donor which is a fluorophore is excited it will emit acceptor if it is a long distance, it does not get affected, but if donor and acceptor are near to each other, then if you excite donor then acceptor will also emit, acceptor will also emit.

And the effect of FRET will be the donor lifetime will be shortened. Acceptor emission will depolarized. Donor fluorescence will be quenched. And acceptor fluorescence enhanced on

donor excitation. So, donor fluorescence will get quenched, acceptor fluorescence will enhance, donor lifetime will be small, and acceptor emission will be depolarized.

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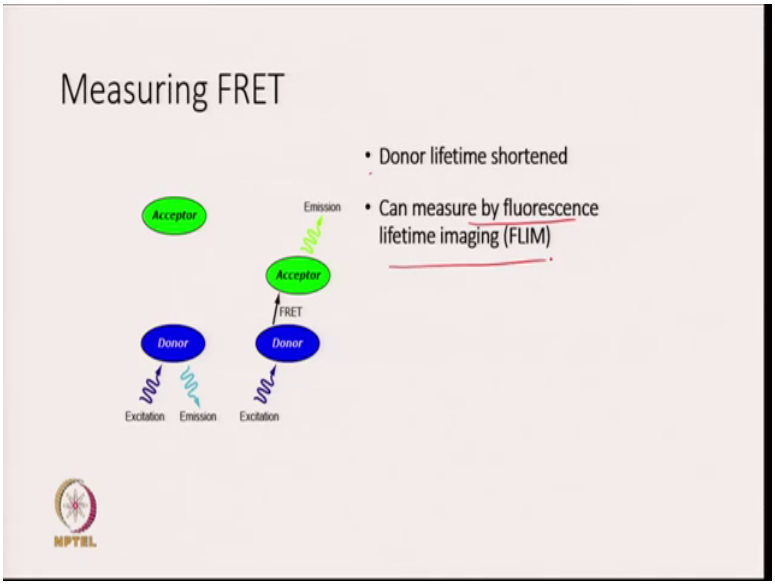

So measuring the distance basically using FRET can be done by using the fact that donor lifetime shortened when donor and acceptor are near to each other. And that is utilized by fluorescence lifetime imaging FLIM to look at the distance between donor and acceptor.

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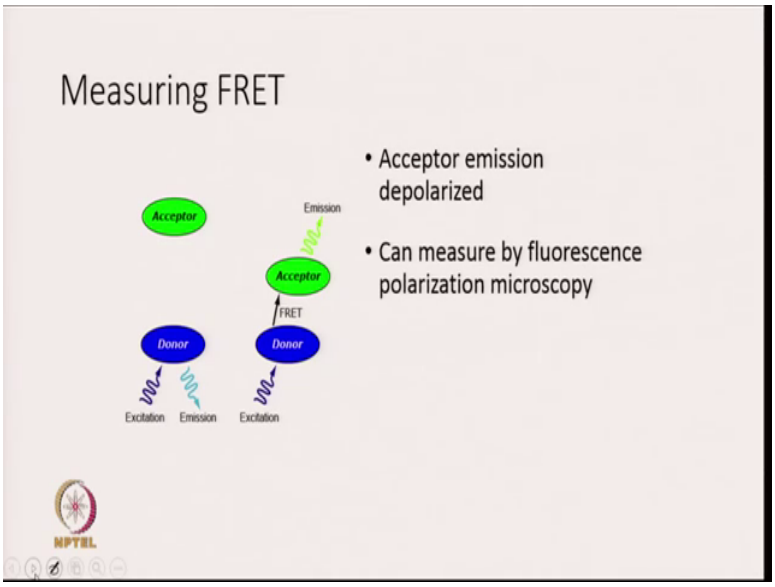

FRET can also lead to acceptor emission depolarization and that can be measured by fluorescence polarization microscopy, fluorescence polarization microscopy and again that can be used to gain information about the distance between donor and acceptor.

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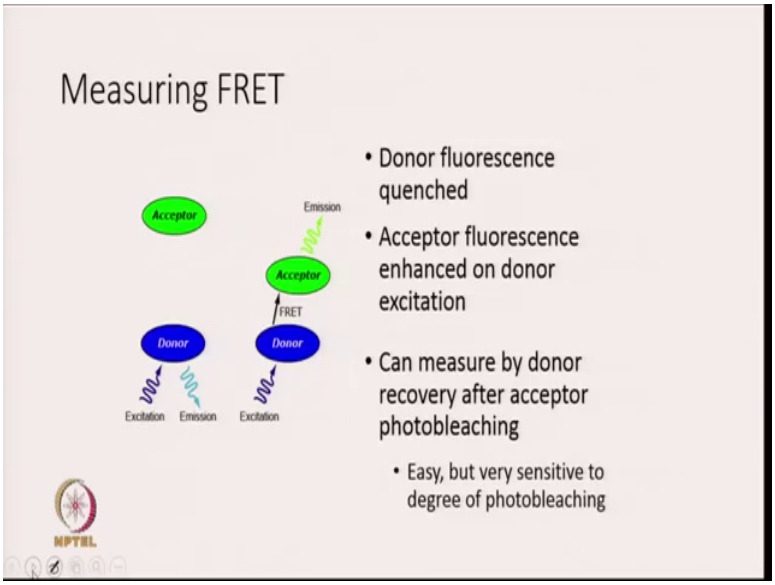

Donor fluorescence quenched. Acceptor fluorescence enhanced on donor excitation. So again we can capture the, okay so let us again talk about this. Donor fluorescence quenched. Acceptor fluorescence enhanced on donor excitation. And so we can measure donor recovery after acceptor photo bleaching and that can also tell you about the distance.

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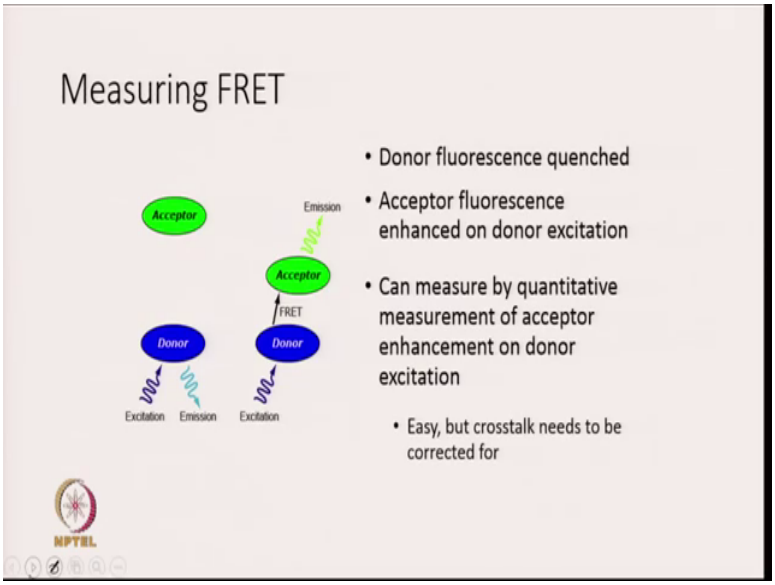

The last one is based on the fact that FRET leads to donor fluorescence quenched. Acceptor fluorescence enhanced on donor excitation. And so we can measure quantitative measurement of acceptor enhancement on donor excitation. It is easy, but crosstalk need to be corrected for.

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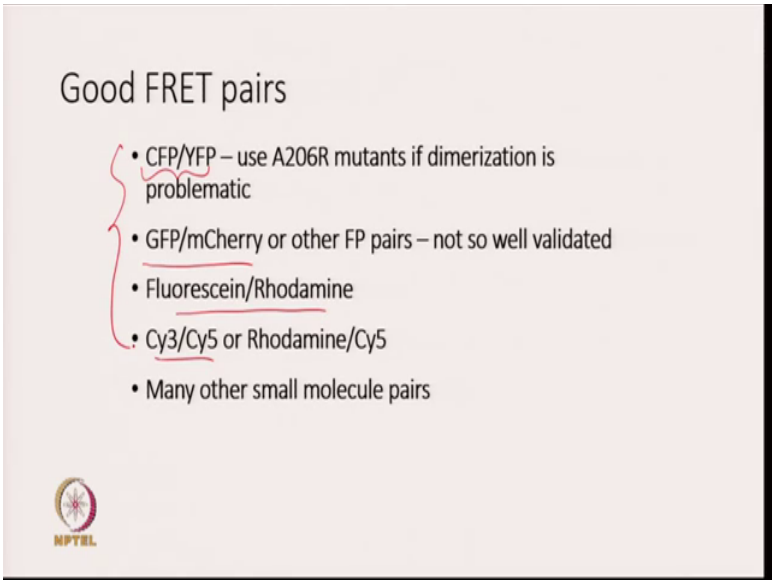

So we can either use lifetime, we can use polarization and we can use fluorescence quenching of donor of fluorescence increase of the acceptor to look at the distance between donor and acceptor. But for that we need a very good pair of acceptor and donor. And generally good FRET pair is CFP, YFP so one is used as the donor and other is used as the acceptor. GFP and mCherry also used at FRET pair. Fluorescein, Rhodamine is used as FRET pairs. Cy3, Cy5 is used as a FRET pair and there are other molecules which has been used as a FRET pair, but these 4 are example of very good FRET pairs.

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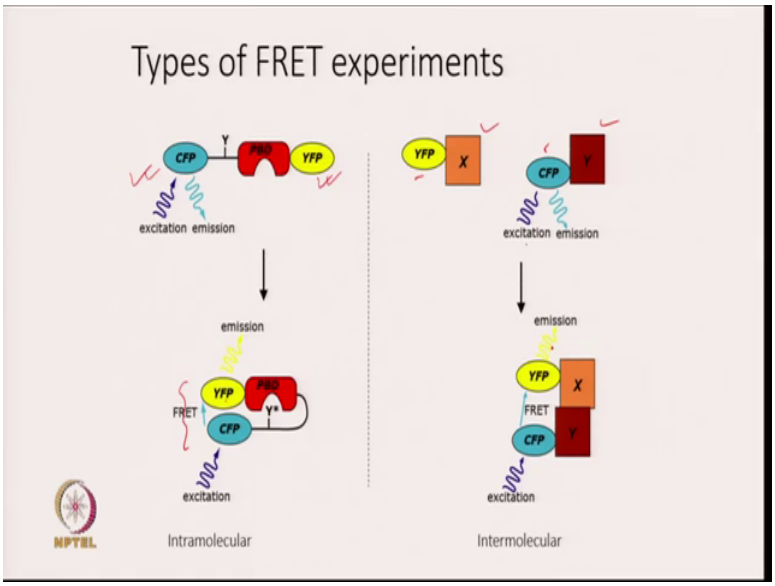

Now there are two different kind of FRET experiments. One talks about the intramolecular distance, another talks about intermolecular distance. For example, if you are trying to look at the folding process, then you need to do this kind of experiments. In that what you do is, you

tag one part of the protein with CFP, another part of the protein with the YFP. And if protein folds this YFP and CFP will come close to each other.

If it is unfolded then if you excite CFP emission will only come from CFP, YFP will not be affected. Whereas, if protein is folded then if you excite CFP, YFP will get affected and it will emit light. So here CFP is acting as a donor and YFP is acting as acceptor, so if they are close to each other then there will be FRET and YFP will show emission.

The second experiment is intermolecular experiment which is generally used to look at the interaction between two protein molecules or two molecules. So these are the two molecules X and Y, one is labelled with YFP and another is labelled with CFP when they are apart. If I excite CFP it will emit. Now, if I put them together and if there is an interaction, it means the distance between CFP and YFP will be small enough to have good FRET and so when you excite CFP your emission will happen from YFP.

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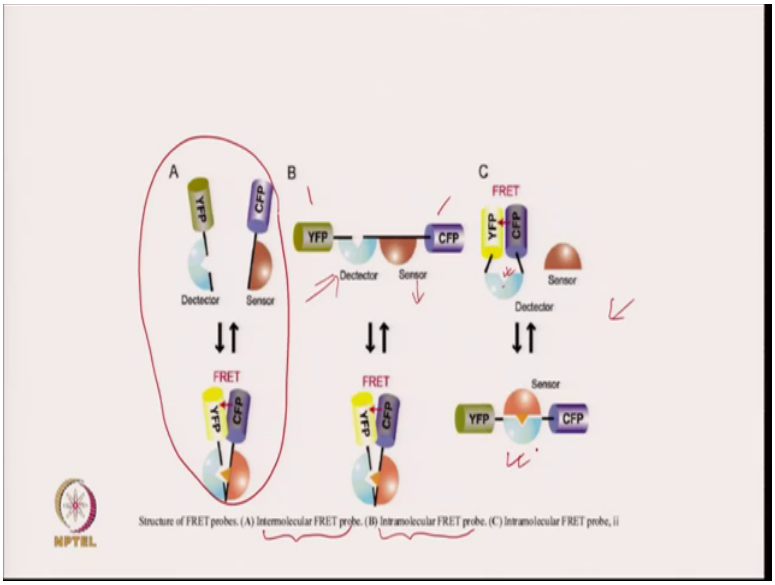

So FRET is generally utilized in 3 different ways, one is by using it as a intermolecular FRET probe, the scheme 1 is given here where we are trying to look at interaction between two different molecules, one molecule which is called sensor is tagged with CFP protein and another which is known as detector is tagged with YFP, what we are going to do is we are going to excite CFP and we are going to detect the effect of FRET on YFP, YFP. If they are far apart we will not see emission from YFP, but if they are near then we can see the emission from the YFP, emission from the YFP and that is how we can look at the interaction between two different partners, two different partners.

The second probe is to look at intramolecular interaction and in that case we will use the FRET pair on same protein, one end will be tagged with CFP and another end will be tagged with YFP, the part where CFP is tagged is called sensor, and this part is known as detector, but we need to again do is we excite CFP and we look at the effect of exciting CFP on YFP.

So, if suppose protein are any molecule in a conformation where these two parts are far apart then there will be no FRET and you will not see emission from YFP, but when they are closed together which generally happens when protein gets folded are a molecule goes into a compact conformation in that case there will be FRET and YFP will show emission.

There is a third case in which sensor is not tagged, this molecule is tagged at one part by YFP and another part by CFP and it is not in complex form, it shows FRET, but when sensor comes and sits on this, sit on this detector the YFP and CFP goes far away and what happens that when you excite CFP, YFP will not show fluorescence, YFP will not show fluorescence.

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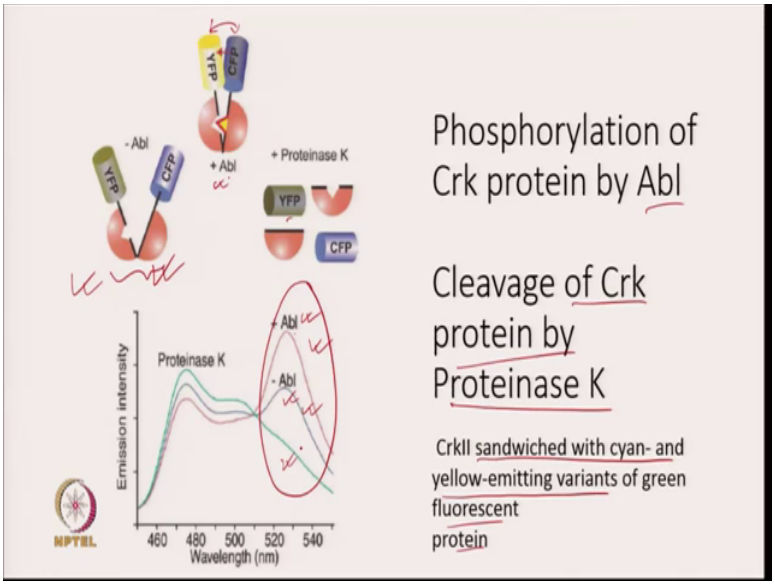

One of the example of using FRET for your signalling process is shown in this example where FRET pair is used to look at phosphorylation of Crk protein by Abl, what is done is a Crk2 protein was sandwiched with cyan and yellow emitting variant of green fluorescent protein, so one end you put YFP and another you put CFP, this is when, this is the structure when, this is the structure when this Abl is not present.

If Abl is present then what will happen that interaction will takes place, phosphorylation happens and then interaction will take place, and then this distance will decrease, this distance will decrease and this distance will decrease and there will be a FRET. So, if you

excite CFP, then YFP will show emission, YFP will show emission, so this is your when Abl is not present and this is when Abl is present.

Now as a control to see that whether this effect is due to coming of, coming together of YFP and CFP, the scientist also looked at what is the effect of Crk protein by proteinase K. So, what happens that if you put protein as K into this Crk2 sandwiched with this sandwiched with YFP and CFP, it will cut the two parts, it will cut two parts and also YFP and CFP will come out.

In that case the distance between YFP and CFP is going to be high and so you expect FRET to be less, FRET to be less and what does that mean is YFP should not show emission, YFP should not show emission, that is what you are looking at this thing if protein is alone, protein is alone.

So, this is your fluorescence intensity when it is, when the spectra is taken in presence of Abl phosphorylation happens, now protein comes into conformation in which YFP and CFP are close in that case your fluorescence increases and when you cut by proteinase K it goes far apart and so fluorescence intensity decreases. So this is a very good example, we will look at the interaction between two parts or looking at the effect of for example phosphorylation on the conformation of Crk protein. So YFP and CFP pair can be used to look at the interaction between two proteins.

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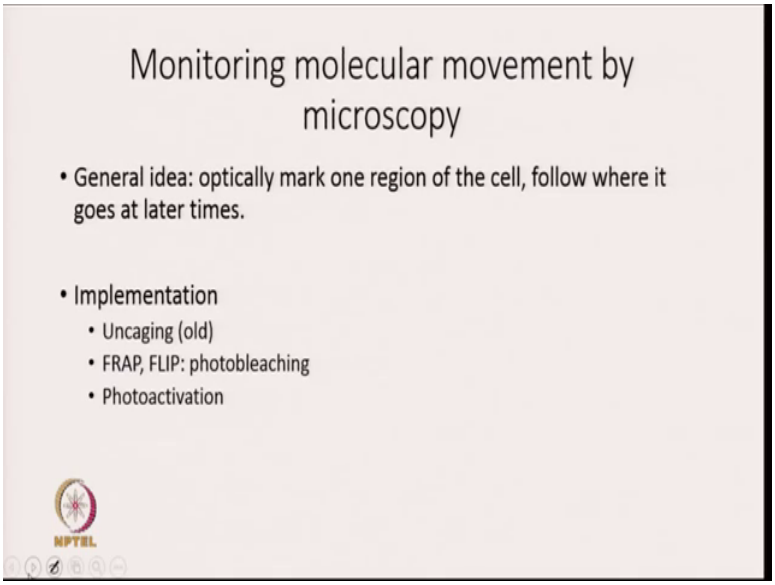

We can also monitor molecular movement by fluorescence microscopy, general idea is optically mark one region of the cell, and follow where it goes at later time. The way we implement it is we can do FRAP, we can use FLIP and we can use photo bleaching, we can also make use of photo activation. So these are the few techniques which can be used to look at the molecular movement by microscopy.

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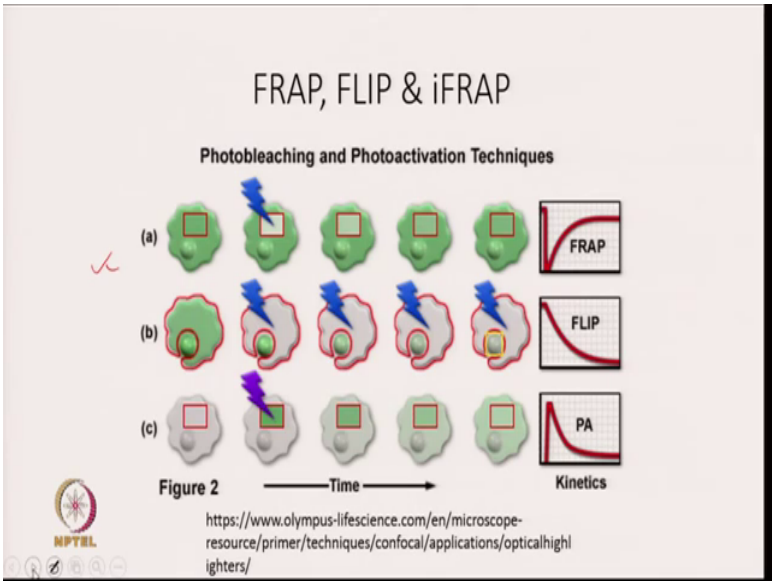

Now let us understand the difference between FRAP, FLIP and iFRAP these are the 3 different photo bleaching and photo activation techniques. So now look at the first case is of FRAP, what you do? You have a molecule stand with dye and now you take a region and then you bleach it with high intensity light.

Now you leave it, what will happen? It will totally bleach when first time it is bleached and now slowly it will recover, recover, recover and that is what you are looking at. So initially we will see this green, then I put the photons this will be deep and then again fluorescence intensity is coming back. So this is one of the technique and that is known as FRAP.

The second technique is FLIP in which what you do is that you bleach the part that which you are not observing, here you have bleached the part which you are observing, here you bleach the part which you are not observing. So for example here and then what will happen? That this bleaching will slowly go to the part which you are looking at and which is not bleached. So you see this green is now decreasing, so effect of bleaching on other part is now seen on the part which is not bleached and that is your FLIP, so you can see it is a decrease.

Now, the third is your what is known as photo activation technique and in this what you are doing is that you are you have unstained molecule, unstained tissue and now what you did is you dye a particular area and what you do is you now put the light. What will happen? It will slowly lose its thing and in turn it will send the colour of dye to every area. And now you can see that initially this and then slowly it is decreasing. So initial this is like we have dyed and then there is a transfer of the dye from ditched area to other part.

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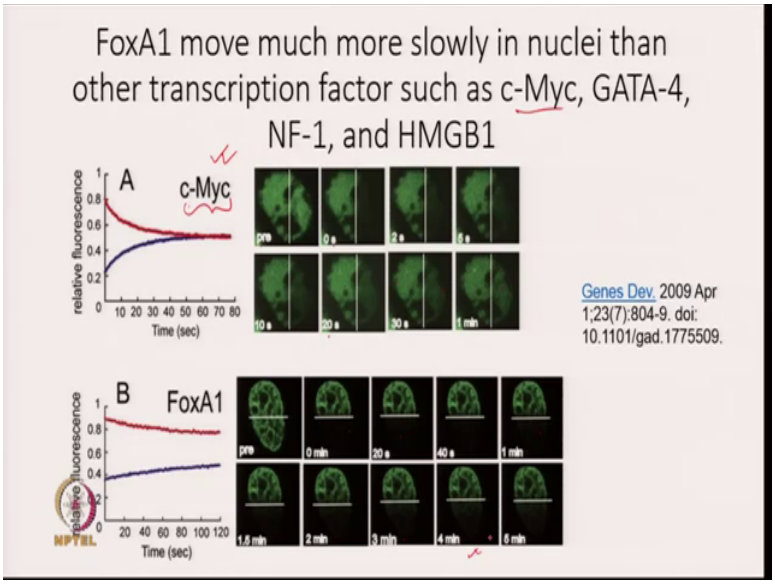

Now these three techniques can be used to look at, these three techniques can be used to look at the movement and I will show you one example. Here in this picture what has been shown is how does FoxA1 moves in nuclei in comparison to other transcription factors. For example

c-Myc. So this is also, FoxA1 is also a transcription factor and what you are trying to look at how does it move in nuclei.

So now look at this, here what has been done that the movement of c-Myc has been seen after photo bleaching, so you can see that this part, this is the pre-treatment and at 0 second you bleach this part and now you see slowly slowly there is a recovery of the recovery of the colour, recovery of the colour.

Now, same thing is done with FoxA1, this half part is bleached at 0 minute and now 20 second, 40 second, 1 minute, 1.5, 2, 3 minutes, 4 minutes, 5 minutes. You can see that in this started looking at the recovered colour at 20 second, whereas till 4 minutes you are not seeing anything for FoxA1, what does that mean is that FoxA1 moves much more slowly in nuclei than transcription factor such a c-Myc. Now you can also look at the relative fluorescence and this is the way it is increasing, the way it is increasing and here you can see the increase is very slow, increase is very slow.

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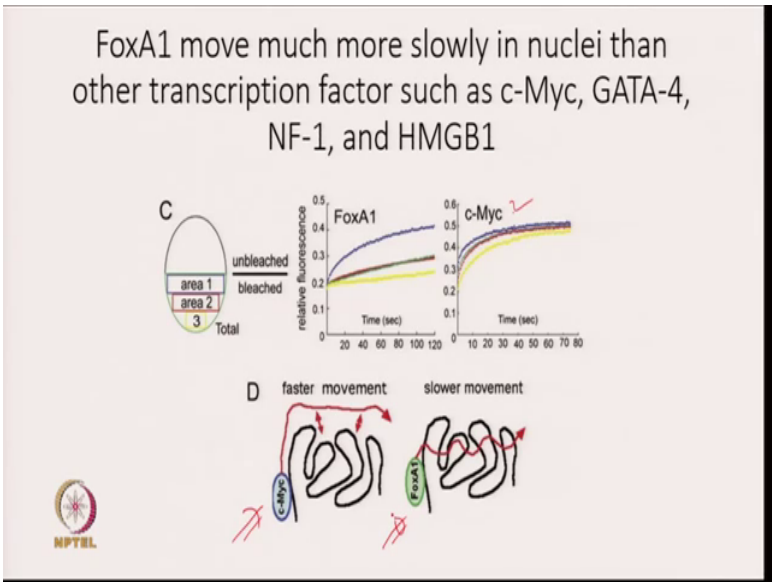

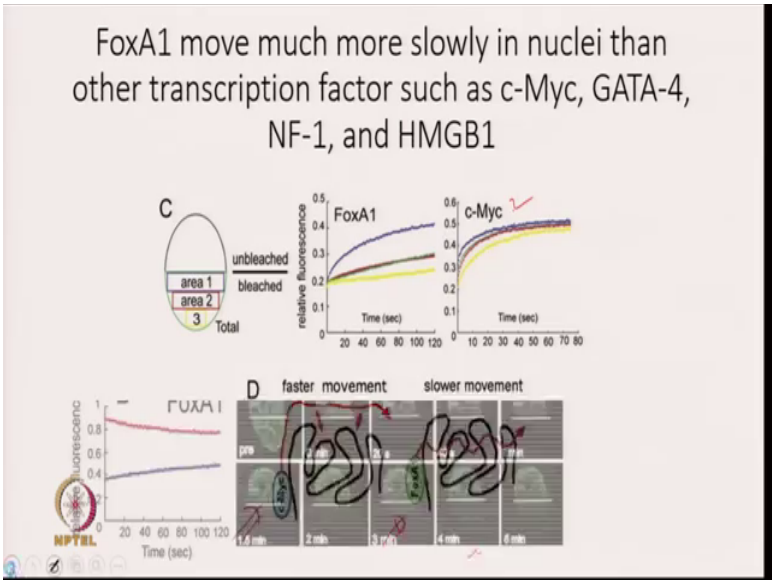

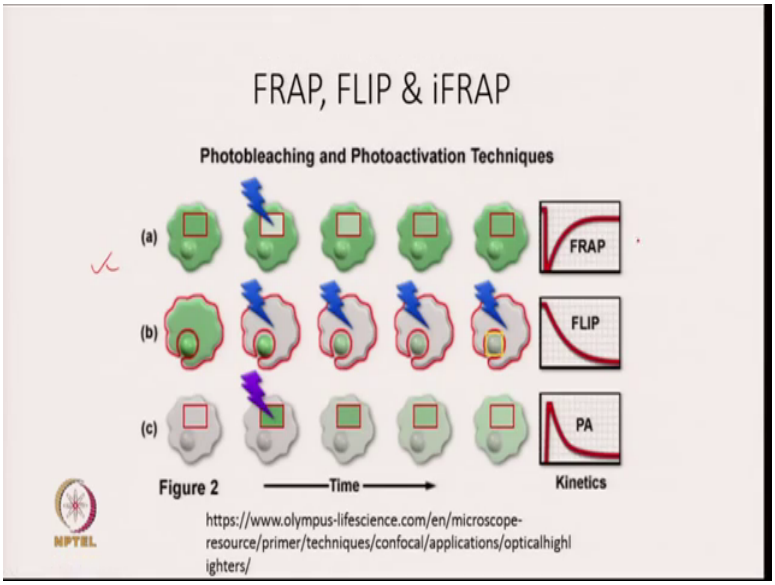

So for FoxA1 increase is very slow, whereas c-Myc increase is very fast, what does that mean is FoxA1 moves much more slowly in nuclei than c-Myc. So this tells you that there is a faster movement of c-Myc compared to the FoxA1 which is undergoing slow movement, so this is one example, several of such examples are present in the literature, so this FLIP of FRAP is generally used to look at the movement. So all these things FRAP and FLIP can be used to look at the movement in a cell.

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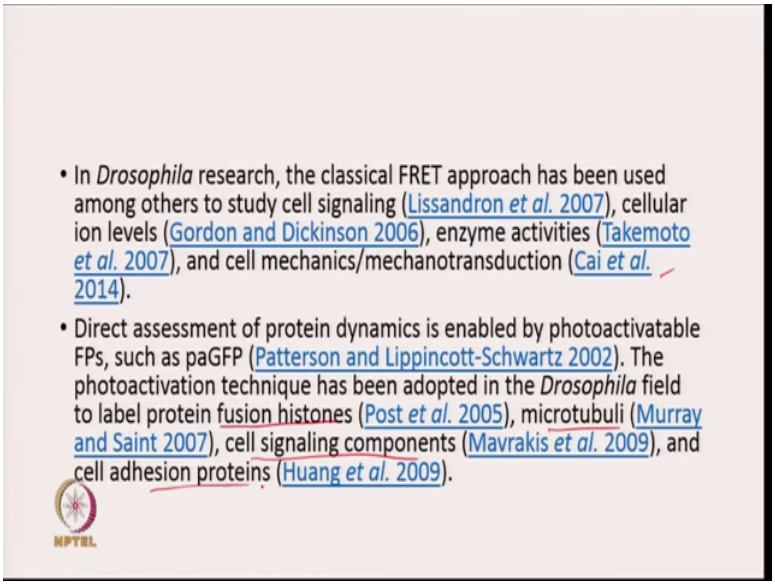

There are several other examples, people have used FRET approach to study cell signalling, cellular ion levels, enzyme activities and cell mechanics or mechanotransduction these are the few reference which represent, you can go and look at it. Direct assessment of protein dynamics is enabled by GFP. The photo activation techniques has been adopted to look at protein fusion histones, microtubuli, cell signalling components and cell adhesion proteins.

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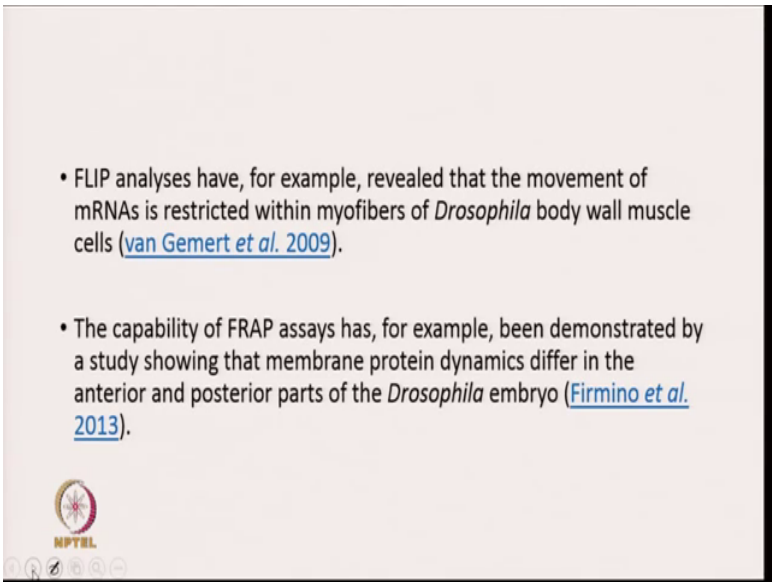

FLIP analyses has revealed that movement of messenger mRNAs is restricted within myofibers of Drosophila body walls muscle cells. Similarly capability of FRAP assays has been demonstrated by a study showing that membrane protein dynamics differ in the anterior and posterior parts of Drosophila embryo. So there are several different kind of application

using FLIP and FRAP is available in literature and one of the major use of these two techniques are to look at the movement.

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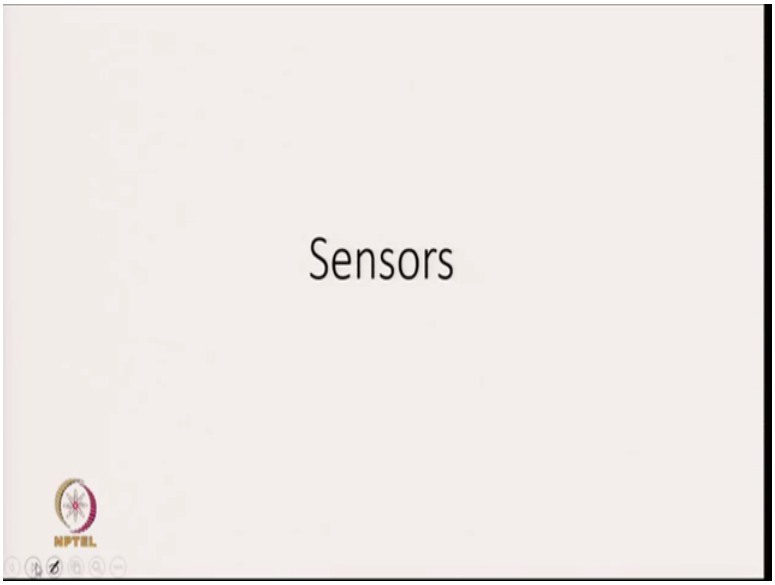

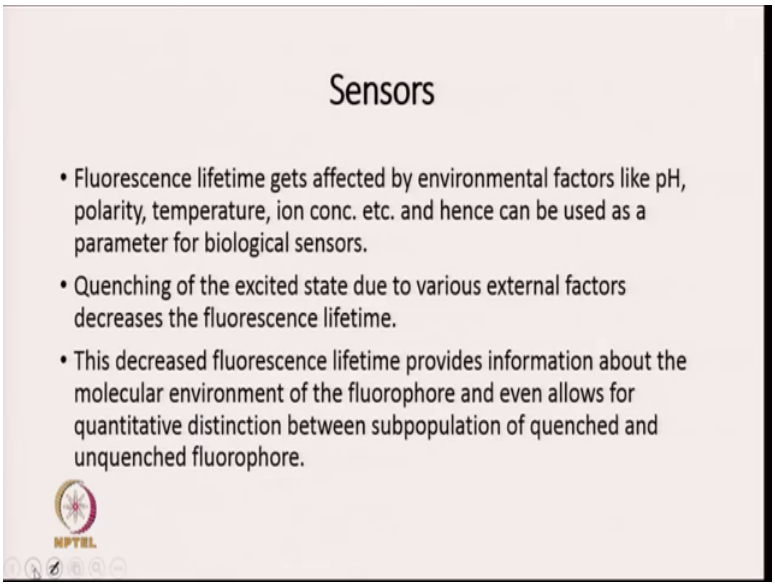

Now microscopy can also be utilized as a sensor and we already know that fluorescence lifetime gets affected by environmental factors like pH, polarity, temperature, ion concentration and what does that mean is pH, ion concentration or temperature is going to affect your images and that is why they can be used as parameter for biological sensing, biological sensing.

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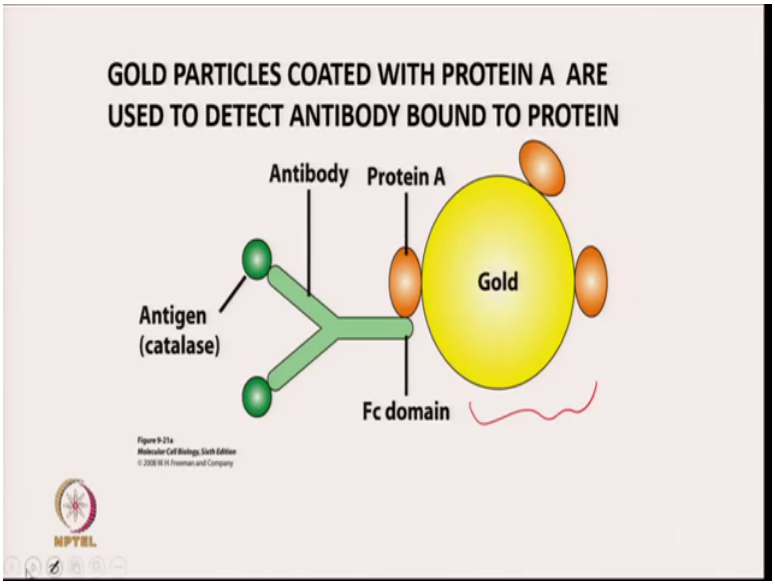

Gold particle coated with protein A are used to detect antibody bound to protein, antibody bound to protein, so what you do is you take a gold particle which is coated with protein A and when you put into the cell it will go to bind antibody and that is how you can detect the antibody bound to protein.

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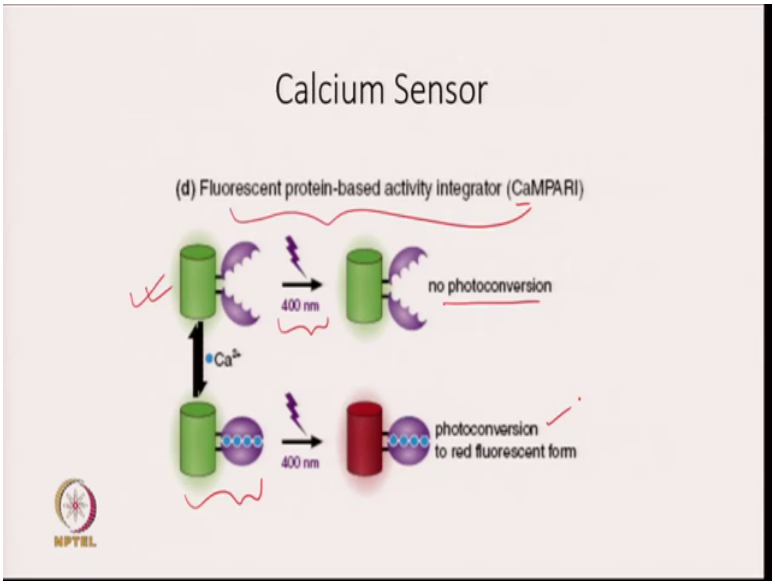

Fluorophores can be used to look at the ion concentration, this is your the protein which has been used as a fluorophore for this is known as fluorescence protein based activity integrator, the name is calcium CaMPARI. So what it does is that this protein has structure like this if I excite it at 400 nanometre there will be no photoconversion, but if I put calcium and when it

goes to hollow form it means calcium bound form, when you excite this calcium bound form at 400 nanometre there will be photoconversion to red fluorescent form.

So, if there is a calcium then it will give you red colour, if there is no calcium then it will give red colour. So just by looking at the red colour or intensity at the lambda max corresponding to red colour you can know what is the amount of calcium 2 plus in the solution.

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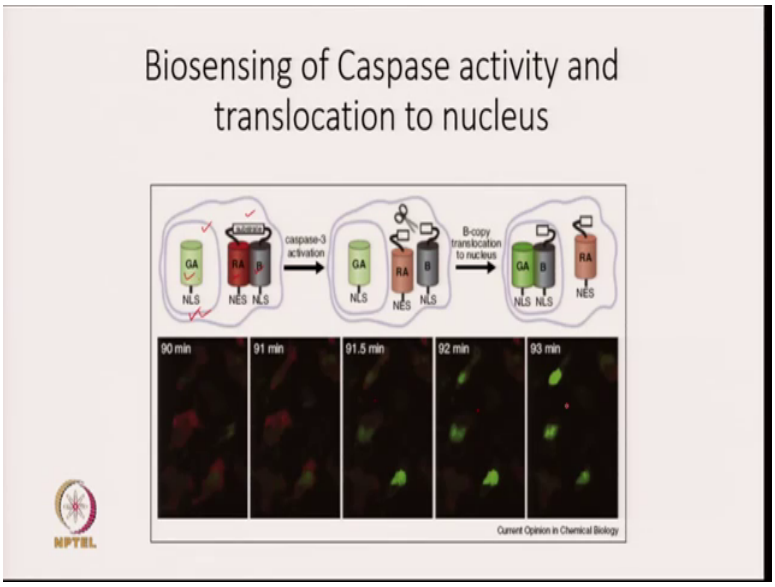

The other kind of sensing which can be done using fluorescence microscopy, there is one example here given that is biosensing of Caspase activity and translocation to nucleus. In this you can see here this is you are here there is RA and B these are the two molecules and there is a GA. If I take Caspase 3 and here is your substrate, if we take Caspase 3 and put it, what this Caspase 3 does, it will cut the substrate and there will be translocation of this B from cytoplasm to nucleus, so this part is nucleus, this part is cytoplasm.

So it is going from cytoplasm to nucleus, and when it goes to cytoplasm to nucleus then it fluoresce to green colour, then it fluoresce to green colour and that is what you can see it here, 90 minute, 91 minute, 91.5, 92, 93 so you can see that colour is changing or this green one is becoming more prominent, green one is becoming more prominent. So, if Caspase is not active then it will not cut and you will not see this kind of image.

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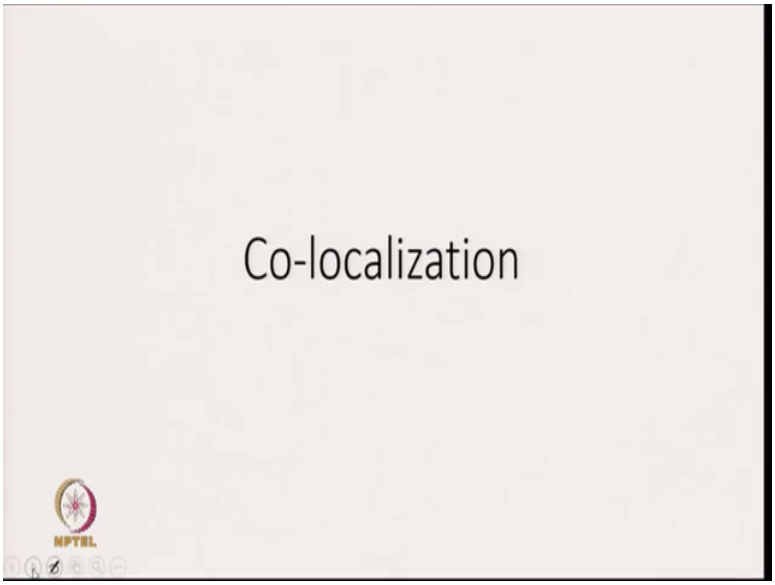

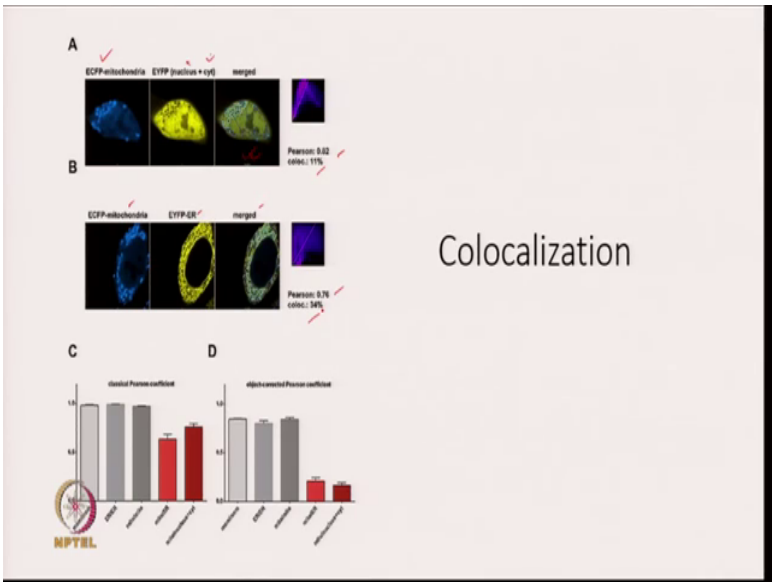

We can also look at Co-localization. One of the example I have discussed in the earlier lecture, previous lecture. So co-localization, here you see ECFP mitochondria, so mitochondria tagged with CFP, this is nucleus plus I took, nucleus plus cytosol is tagged with YFP and when you merge it will be like this and Pearson factor is 0.82, ECF with mitochondria are this is the merge thing and what you get a Pearson factor 0.76 and co-localization is 11 percent and this here co-localization is 34 percent.

So there are way to calculate the co-localization from the merged picture and the image to find the percentage of localization factor, I am not going into detail but if you want you can go and look at some of the book I have referred to.

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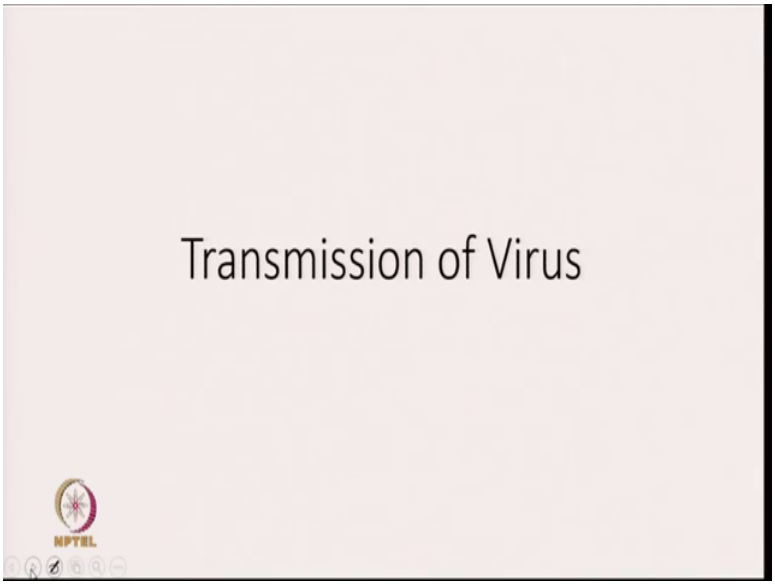

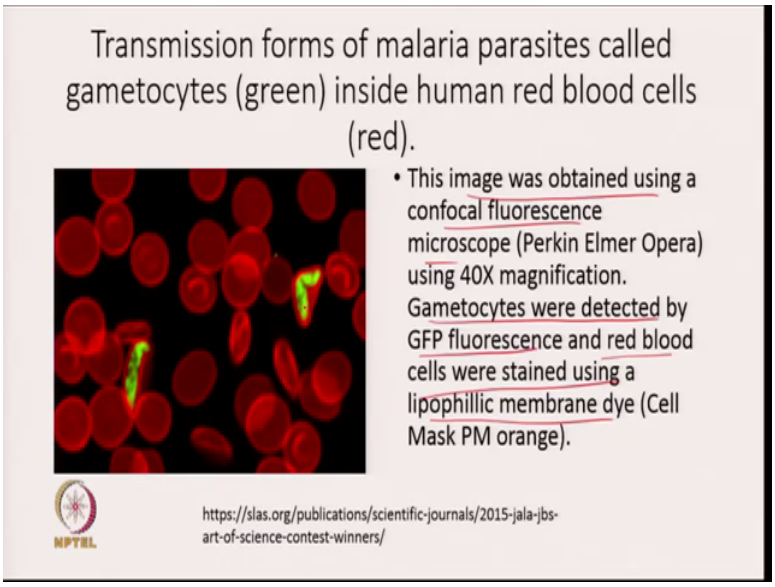

We can also look at the transmission of virus using fluorescence microscopy and this is one of the example where transmission forms of malaria parasite called gametocytes which is green here has been seen inside human red blood cells, human red blood cells. So this image was obtained using confocal fluorescence microscope. Gametocytes were detected by GFP fluorescence and the red blood cells were stained using lipophillic membrane dye, lipophillic membrane dye. And now you can see here that you know you can look at the gametocytes of the malaria parasites, how it has gone inside human red blood cells.

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We can also know internalization of the different bacteria or different molecules inside the cell. For example, in this case what you are going to see is amoebae internalize human cell fragments, preceding human cell death. Now you can see here phase this is A H, now you can see this, if you merge it, it will be like this 28 minute now you see here there is a leak internalization, here you can see internalization, here you can see internalization and that can be seen in a much better way using some other dyes, some other dyes. And so fluorescence microscopy can also be used to look at internalization of bacteria or virus inside a cell fragment.

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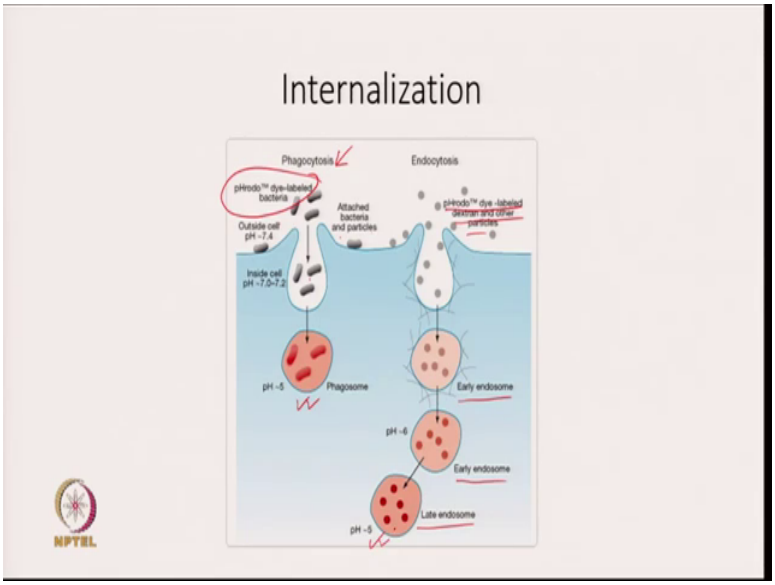

Here is another way to look at the internalization for example, during phagocytosis if you take this dye labelled bacteria, this is the attached bacteria and the particles, outside cell it is pH 7.4 it will not fluoresce, if you go inside the cell and if it goes in the phagosome where the pH is 5 it will start showing fluorescence, it will start showing fluorescence.

So you can look at the internalization using fluorescence spectroscopy. Similarly, this dye labelled dextran and other particles is here and if they go to early endosome they will change colour like this, early endosome their colour is this and late endosome you can see it is quite intense rate, quite intense rate. So by using P A sensitive dyes, by using P A sensitive dyes you can look at the internalization, internalization of bacteria inside the cell.

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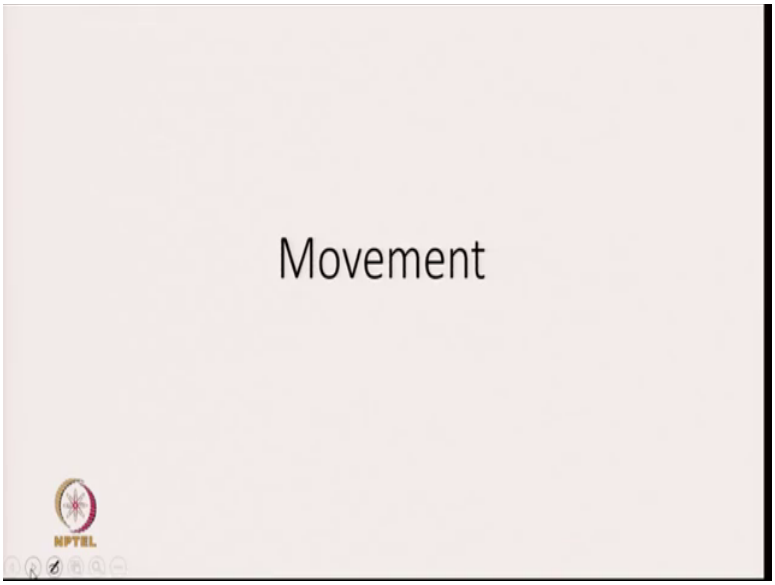
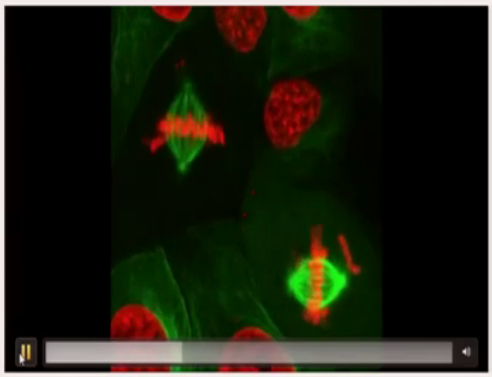


Observing Mitosis in Epithelial cell

https://www.youtube.com/watch?v=TKKdxzHVK2w



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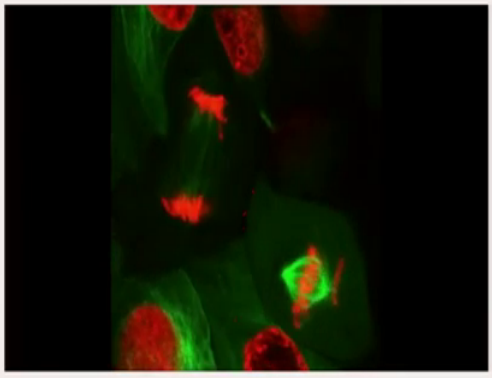

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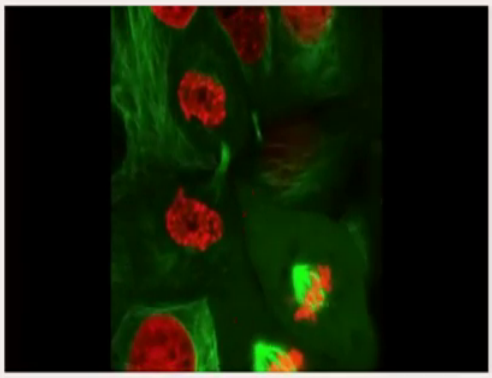

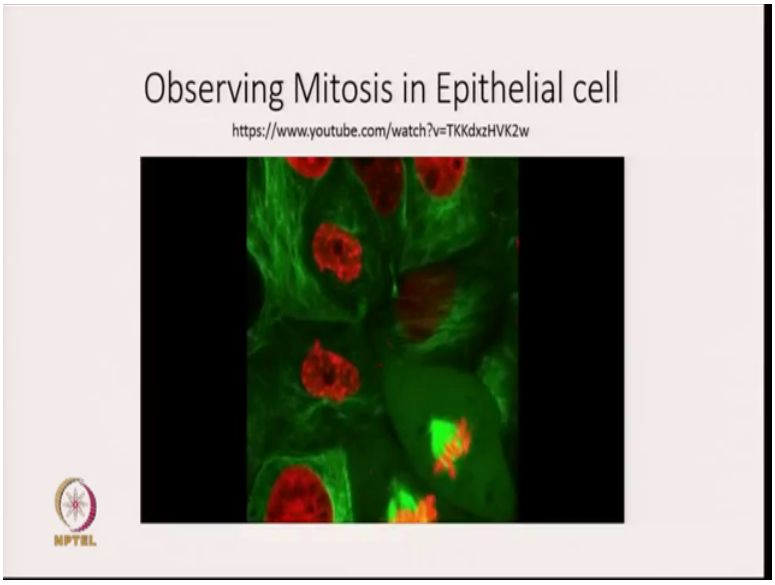

We can also look at the movement, I have discussed few of the example, what I am now going to show you a picture in which fluorescence microscopy is used to look at the mitosis in epithelial cells, mitosis in epithelial cells, just look at this, now you can see the picture. Let us see it again, how beautiful it is.

So you can see how important fluorescence spectroscopy is in looking at the various even inside our cell, inside our body. So it has many more applications, I have tried to show you some of the applications, I hope that will give you a flavour of how fluorescence microscopy can be used, how fluorescence microscopy can be used. So thank you very much, see you in the next lecture, bye.