Biophotonics
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Module 06: Bioimaging: Principles and Applications
Lecture 29: Fluorophores and Fluorescence Microscopy Techniques

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Welcome back, bioimaging is a very a large field and an entire course can be made out of bioimaging and I know you are thinking that in the previous class I went overboard and I went more than my stipulated time. Here I will try to ah amend and I will try to make it within 30 minutes so that I can hold off your attention span.

So, today's course, today's class I am going to discuss about fluorophores and fluorescence microscopy techniques. This is also going to be quick because I believe the fluorescence part was already discussed, you now know, I assume you now know what fluorescence is and a little bit of fluorescence microscopy has also been discussed.

So, let us go on with it, we are mostly going to discuss a little bit about fluorophores. Fluorophores are those chemical you know about chromophore, I have discussed what chromophores are, fluorophores are the fluorescent equivalent of chromophores, chromophores get subjected to light, they react based on the light, they determine the color, fluorophores determine the fluorescence property of the material.

So, if it is a large complicated molecule fluorophore can be that molecule, that part of the molecule, that fluoresce, fluorophore can be individually as themselves, you will see or certain section of a large molecule can fluoresce by themselves or you can have an individual molecule, you can create a molecule by itself which when excited by light will fluoresce, will emit or will give you the fluorescence effect.

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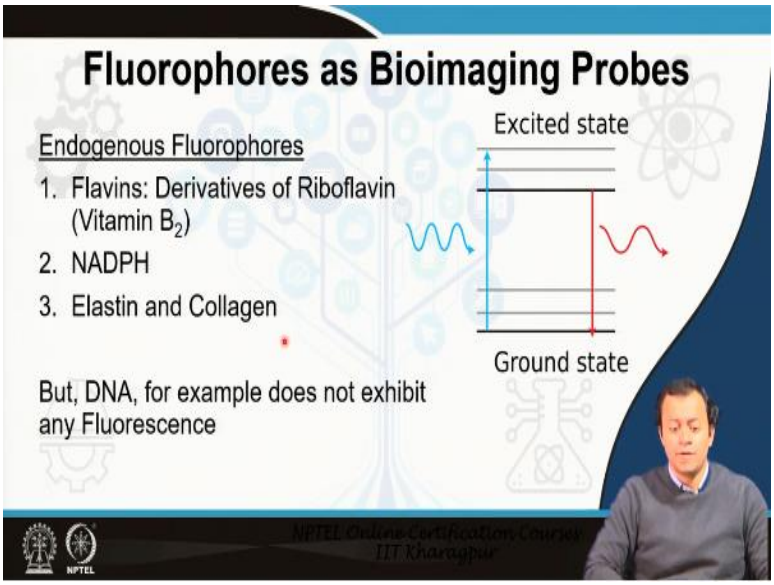

So, there are several materials within the biological matter, within the biological body that are endogenously fluorophores. I discussed this about it, you shine light into them the electron goes from ground state to excited states, then it returns back to the ground state losing some of the energy in the process by non-radiative loss, non-radiative radiation, phonon heat what not and the output of the photon is less than the input of the photon, the energy output, energy of the photon is less than the input energy of the photon.

So, you excite by higher energy photon you get lower energy photon coming out and this is the basic of fluorescence. So, several of biological matter, several parts of the biological matter already have an auto fluorescence, as I said they have some amount of fluorophore associated in their entire bigger chunk and that releases upon excitement by specific amount of photon, they release some amount of photon.

For flavins which are part of riboflavin, vitamin B2, NADPH, I discussed this elastin and collagen and hence, you saw that microscopy, the second harmonic generation microscopy where collagen was shown, but not all biological molecules or not all biological materials has fluorescence, all biological materials fluoresce.

For example, DNA is quite important we need to understand DNA to understand gene, we need to understand DNA to understand gene, we need to understand gene to see how the gene expression is happening, what are the proteins that are being created, synthesized and from the protein we need to know how our bodies are functioning. So, if we are unable to see DNA, then what is the point.

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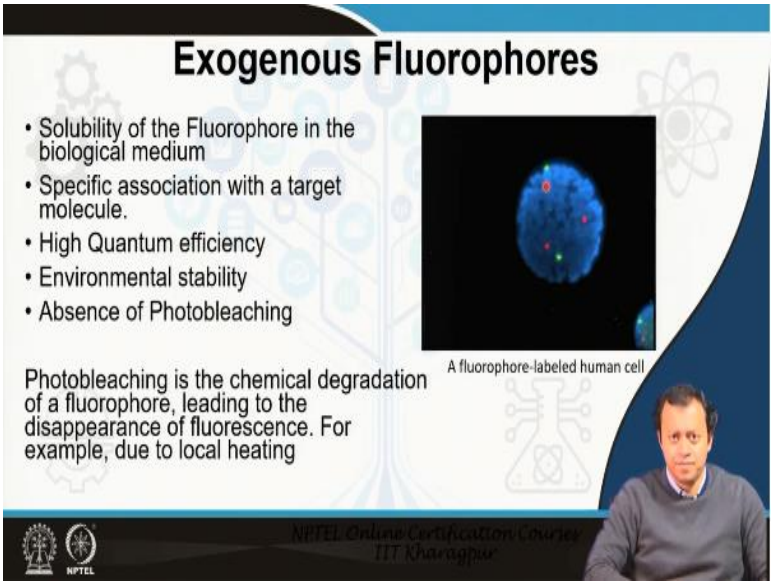

Therefore, what we do is we generate molecules, we generate molecules, exogenous molecules, we generate materials, we generate substances that could be made to attach with the biological matter that are we trying to look into, trying to measure, trying to image, trying to detect, trying to ascertain. So, we create certain fluorophores, we create certain molecules that needs to get attached to the area of our interest, we call this process labeling.

Just like you label with your highlighter pen onto some part of the text, so that that part of the text become highlighted hence, the term highlighter, yes, that part of the text become

highlighted. So, of all the different areas of the text, all the different areas of the paper, of the different areas of the paragraph you can immediately look into it, read it, understand it, memorize it everything, we try to do the same thing, I remember discussing this but let this be a refresher course as well.

There are certain properties that we need to be associated with fluorophores, they need to be soluble and stable at the biological medium, there is no point if it is unstable, they have to be very, very selective, so the molecule has to attach at specific regions of the cell, this is a fluorophore level human cell, this fluorophore, this exogenous fluorophore that fluores, that molecule that fluores that, emit that that shows fluorescence has to attach at specific areas only, not randomly attached anywhere and it should have obviously high quantum efficiency and environmental stability and absence of photobleaching.

What is photobleaching? Photobleaching, I thought I taught you quenching, it is similar to that is the chemical degradation of the fluorophore after certain time, the fluorophore destroys itself because of local heating, because of interacting with other molecules and it no longer fluores.

This, is a perfect example, this is a human cell in which we have labeled it with red type of fluorophore and green type of fluorophore i: e molecules which emit red and green light upon excited by I think blue light and these fluorophores attaches themselves only, only with specific types of chromosomes.

They are not randomly attached, so wherever they are attached, wherever this emission is coming out, you can understand that there is a chromosome and thereby you focus your light, focus your microscope onto it do whatsoever confocal microscopy, laser scanning microscopy this, that, whatsoever and life is good you are able to image it beautifully, laser scanning confocal microscopy you do it here.

And how much super resolution you will get, especially, especially if you bring the objective of your lens within 50 nanometers of that particular sample, what do you think

you will get, can you do that? Now, remember 50 nanometer is a run of the mill term do not always quote me for saying 50 nanometers.

I have got it for at a 60 nanometer or 65 nanometer or even by 50 nanometer it is not coming I want, I had to go to 20 nanometer or I got that sample beautiful image at 100 nanometer, depends on the sample, it depends on the wavelength that you are looking for, it depends on how strong your evanescence field, it depends on several different features especially aperture, the distance etcetera.

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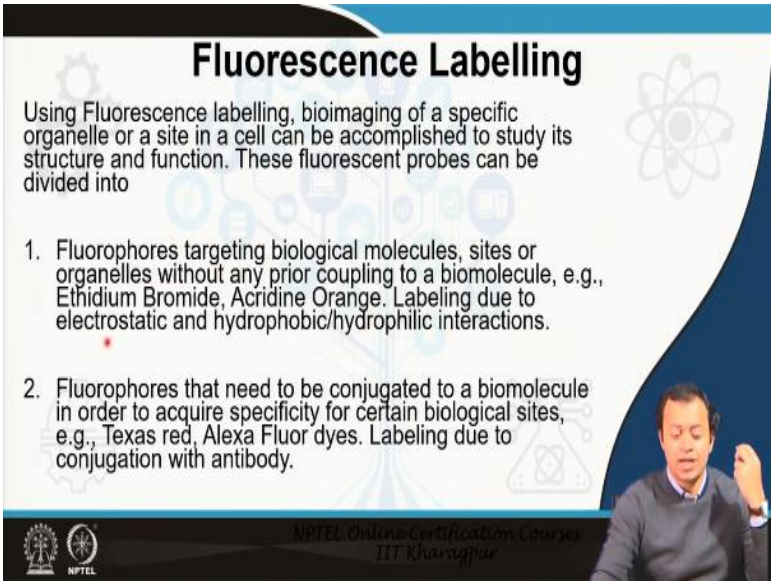

So, what is fluorescence leveling? As I said a bioimaging of a specific organelle or a site of a cell that can be accomplished to study its structure. There are two ways these fluorophores can be made to label, can be made to attach, can be made to highlight a particular area, they can attach with a specific site using electrostatic or hydrophobic hydrophilic interaction.

They can attach themselves to that or they can attach to another biomolecule and that biomolecule then get attached to the particular material that you are trying to image, that you are trying to understand, this is direct, this is indirect, here the fluorophore gets directly attached using electrostatic or hydrophobic hydrophilic interaction, here they

attach first with the biomolecule, then the biomolecule have some way to get attached into the biological site.

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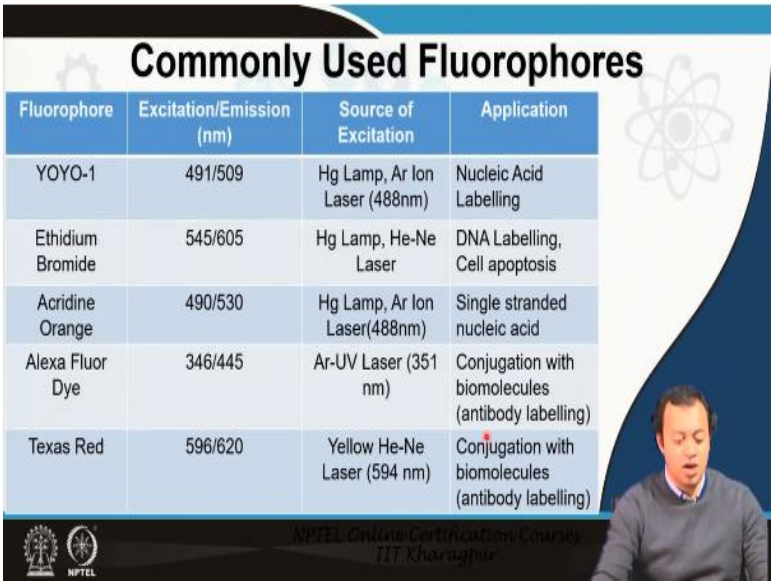

And here is the list of several of such fluorophores YOYO-1, Ethidium Bromide, these are highly, well not highly but several companies make these kinds of materials, make these kinds of fluorophores and they have special way to attach it to a specific location within the biological material, they spend a huge amount of time, energy and money to develop these materials and they are sometimes classified.

They can be used for nucleic acid leveling, they can attach themselves to a specific, specific part of the DNA thereby that area fluoresce and that area of that could be imaged using different argon lamps. So, this table is something that you should look into to understand but this is not the important part I am actually like the vision part, I am itching to show you something uber cool.

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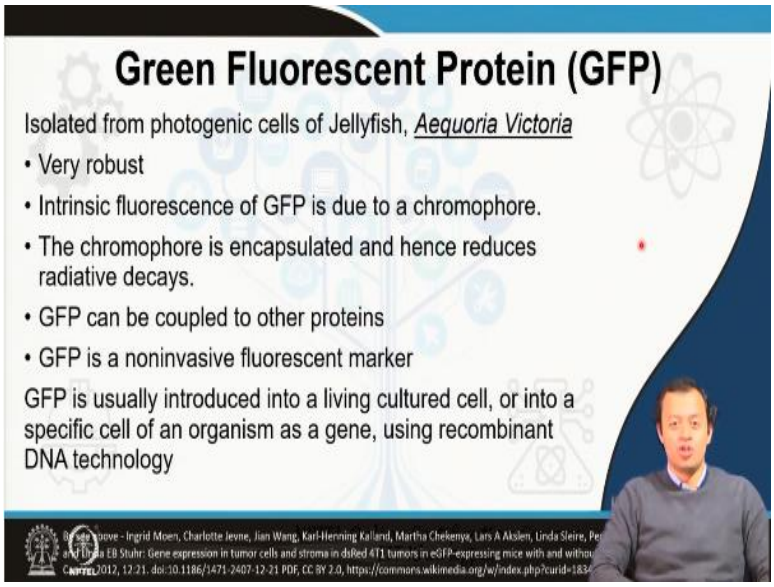

And that is GFP, Green Fluorescence Protein, this has revolutionized fluorescence microscopy when it comes to biological matter and this is uber cool, this is so, so cool I cannot even try to explain it to you. So, what is green fluorescence protein? Green fluorescence protein is a protein that fluoresce, and it has been extracted from jellyfish, this is the scientific name, I cannot pretend to tell you that I can pronounce it correctly, jellyfish, jellyfish will do.

So, they extracted a protein from the jellyfish, this protein is very robust, intrinsic property etcetera, etcetera you read it at your own time, what I want to show you is that this is a protein, this is a biological material, your DNA makes protein, we do a process called recombinant DNA technology, you can consider transfection, biological student know what I mean by transfection, CRISPR and what not, CRISPR is going quite far away.

Recombinant DNA technology transfection you can enter, you can make this DNA go inside your DNA and the overall gene, your gene gets modified because of the foreign gene that is coming up and then your DNA will express or will synthesize a protein that can fluoresce, you know about genetically modified food, huge debate is going on, would you like to inject foreign DNA in your body, do you already not inject foreign DNA,

RNA in your body, when you eat food, eat protein if you doubt me ask that gentleman who ate supposedly uncooked bat at a far corner in China and the pandemic started.

Supposedly, I do not know if it is true or not but that is what is going on, when you get a vaccine what do you think is entering in your body, meaning if GFP, green fluorescent protein enters your body, that GFP gene enters your body, your body's DNA gets slightly modified and your body start generating green fluorescent protein, this is a fluorescence protein, meaning, when light will shine on certain part of your body it will fluoresce.

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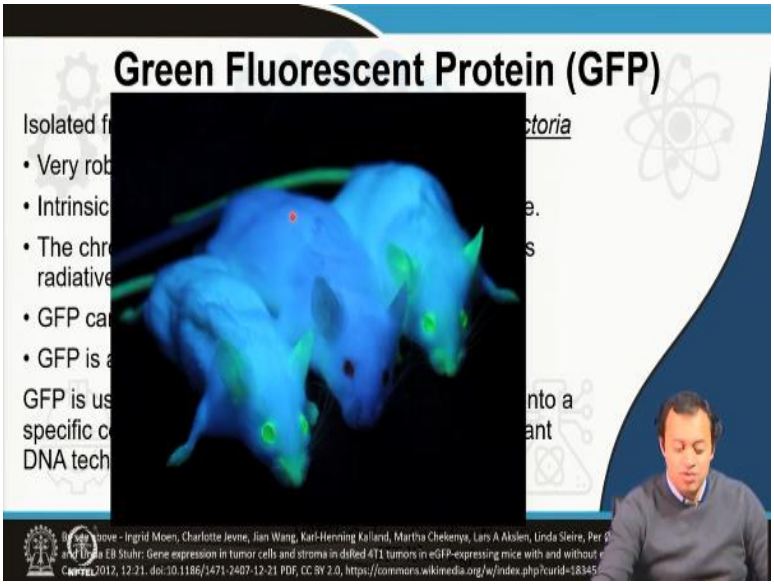

Do not believe me? Check out this picture, the bottom, the middle mouse is normal mouse, these are the two different mouses, where they have got tumors inside their eyes and different parts of the body, GFB has been made to attach with that tumor, the DNA has been made to attach with that tumor and that tumor and when they are put in ultraviolet light they are fluorescing, when put under ultraviolet light they are fluorescing.

If this can happen inside mice, can it not happen inside you, would you like to add some of GFPB gene in your body which will look as normal as anything but as soon as you enter a discotheque, certain part of your body starts glowing like that, imagine how cool that is going to be. Would you like to do that?

Would you like to genetically modify yourself? You already are perhaps genetically modifying yourself, you are already ingesting foreign DNAs as I gave you the example of the pandemic, how the pandemic started, maybe again disclaimer I do not know this is something that came up in news media but in these days of fake news, we do not know what is correct, what is not correct.

But you understand where I am going with it. So, green fluorescence protein has changed the overall mechanism, why, because you enter a DNA, you enter a protein, you enter a gene, not a protein, you enter a gene or you enter a DNA inside the body that DNA gets attached to a specific area and start synthesizing proteins.

So, all-natural processes and then you get this, is this not natural the body of the mouse produced this protein which fluoresce, so why is this unnatural, why is this unnatural? Imagine how far our engineering, photonics, genetics and nanotechnology is going would you like to do that to your own body, to say for something completely different, not for fluorescence but say grow an extra ear?

You can CRISPR technology is completely destroying this is thalassemia, these genetically disease, sickle cell anemia we have a huge amount of sickle cell anemia in the central Indian belt, Chhattisgarh, Orissa, Madhya Pradesh, Jharkhand all these areas suffer from this genetic mutation which causes people living in those areas, ethnic people living in those areas to have sickle cell anemia and that is a genetic disease.

There is no cure but you can genetically modify it, you can genetically modify it, you can put a DNA which will stop making sickle cell, blood cells RBCs and thereby prevent these genetically blood borne diseases like thalassemia, sickle cell anemia etcetera. Obviously, we are trying to prevent it, congenital blindness, several babies are born without the ability to see, congenital is a gene we can cut and paste that gene.

But at the same time, we can do several untoward, untold or unspeakable things with genetic technology as well. It is up to you, it is completely up to you, nuclear power you can drop it on Hiroshima, Nagasaki or you can light up an entire city and thereby make your life easy, up to you.

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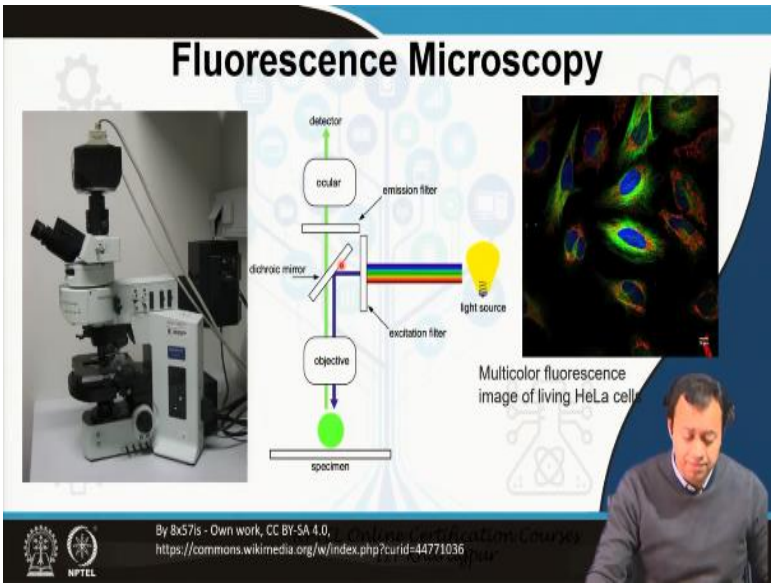

So, fluorescence microscopy quickly go through it, I am getting tired by teaching the same thing over and over, this is repetition understand, you have a specimen, there is a light source, we have an excitation filter, a particular light is being made to fall, this particular light beam is absorbed by the specimen and the specimen starts to fluoresce and we use a filter, emission filter to just take that particular light out, that particular light out, these are all those detectors and filters and what not and we are able to see beautiful images.

And obviously, we add several different types of fluorophores, we shine light either continuously or at specific period of time, pulse lasers can be done, different types of pulse laser can be done, different types of fluorescence red, green, blue etcetera can be done, act simultaneously or at different time, all of these permutations, combination can be done. Nowadays, they are doing a 3d emission just like you saw in OCT shining light from here, shining light from here, shining light from there, so you get an overall three-dimensional image of fluorescence.

This is a HeLa cells, biological student know what HeLa cells stands for cancer cells, Henrietta Lacks donated her cell, so this is a cancerous cell and different areas, so fluorophore, different types of fluorophore got attached to different parts of the cell, so

green fluorophore got attached to the cytoplasm, blue fluorophores got attached to the nucleus, red fluorophores got attached to I think some area within the cytoplasm and you send different types of pulse lights, one at a time or simultaneously and then use different types of filters to filter different light.

Again simultaneously, or one at a time and then you reconstruct the entire image and by doing all those dark field, bright field bringing this closer, bringing this further you can get a super resolution microscopy laser scanning, super resolution microscopy where only one single molecule is being detected, it is possible, not by me I cannot do that I have tried and failed and this is one of my regrets in life, but some people do it.

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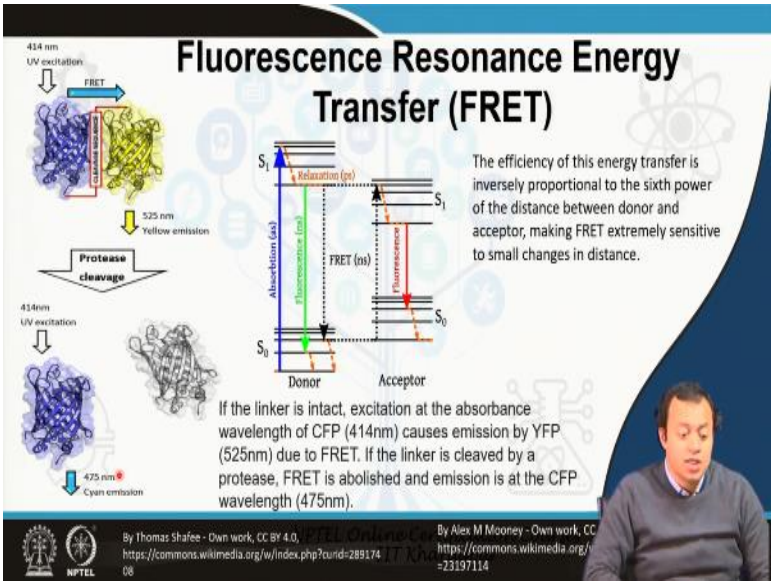

We discussed about the Fluorescence Resonance Energy Transfer as well, remember the donor and acceptor part, if the donor's emission falls within the absorption of the acceptor, then remember transferring where the electron instead of going from the LUMO level of itself to the HOMO level of itself, it gets transferred to the LUMO level of the acceptor. So, there is a transfer of charge, the electron instead of going here returns here, we have discussed this, we have discussed this.

So, but this thing only happens when the donor and acceptor are very close to one another. So, we utilize this fluorescence resonance energy transfer imaging to see protein

protein interaction, if two proteins are close to one another there will be different types of emission because then this will fluoresce, acceptor will fluoresce, if the distance between them are apart, then the donor will fluoresce, there will be no charge transfer, the charge transfer can happen only when they are closed.

Example is this, you have a cyan protein and you have a yellow protein, if they are connected using this protease cleavage, using this protease molecules FRET takes place and after 414 nanometer UV excitation you get an output of 525 nanometer, if this cleavage, if this handhold, if this protease which has connected between two different proteins is broken.

There are two separate things, you try to give the same exact UV excitation and you get 475 cyan emission which is also blue, if they are the proteins are together i: e donor and acceptor are connected you have a yellow emission, if by some time the protein is no longer interacting, if they have gone their separate ways, separate ways you have a blue emission.

So, looking at the same protein you can understand whether they are close or they are separate, whether they are interacting or not interacting, whether the protease is present or it has it separated.

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So, I think I tried to finish it within 30 minutes, these were the concepts covered fluorophores, fluorescence labeling, GFP, GFP is uber cool please, please read about GFP, obviously, if you have green fluorescent protein obviously, several deviations, several derivations from green fluorescence protein has been made, you have yellow frozen protein, red fluorescence protein what not and yes.

I am looking forward when I can inject all of them into my body and I will be that illustrious man as Ray Bradbury said and yes, fluorescence microscopy and fluorescence energy transfer, these are the topics that I mentioned.

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And this is my reference, I have become a fanboy of professor Paras Prasad after studying his introduction to biophotonics part, I took hold of his nanophotonics book as well and it is equally fantastic and if you want to understand fluorescence, there is no better book than Joseph Lakowicz, Principles of Fluorescence Spectroscopy, try to get that.

So, thank you very much, we are beginning at the end of the module of bioimaging, in the next class I will show you some of my own work in bioimaging and that will break the diffraction limit. Thank you very much.