Nanobiophotonics: Touching Our Daily Life
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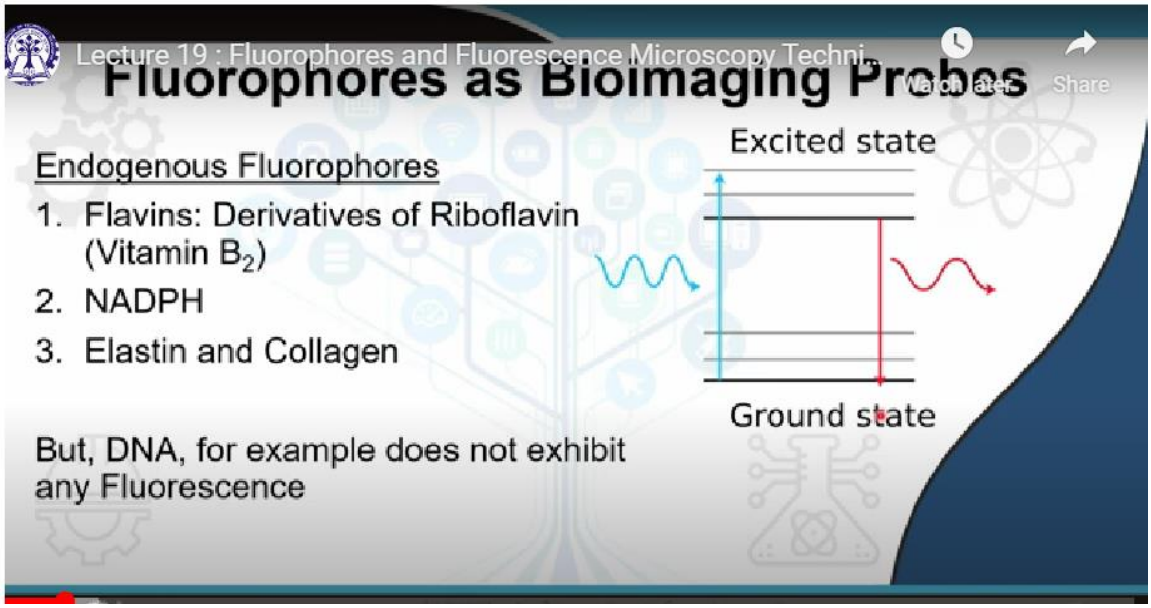
Lecture No. 19
Fluorophores and Fluorescence Microscopy Techniques

Welcome back. We are ah in the middle of the discussion about the bio imaging of um nanobiophotonics topic. In the previous lecture, we saw about some basic imaging principles and how ah it is not always the quality of the instrument that matters, it is the diffraction limit that matters. Today, we are going to discuss about one very specific ah bio imaging technique as come as as relevant to biophotonics and that is ah ah ah fluorophores and fluorescence microscopy right. So, welcome right. We have discussed fluorescence in previous classes you know that these are those materials which very thick homo and lumo bands, you ah shine them with a high energy high frequency photon.

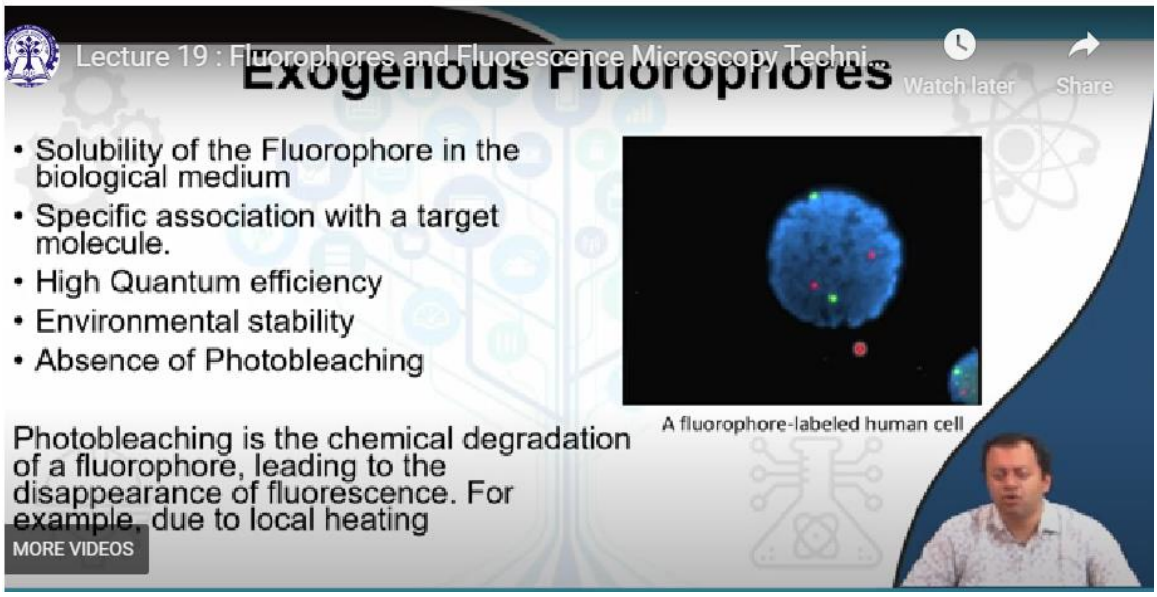


The electron absorbs the photon goes from a ground state grows from a lower energy state to higher energy state. At the higher energy state, it undergoes from non radiative transition and then it returns back again to the ground state by emitting another frequency of photon. So, blue light can convert into red light. This is the very basic the loss is happening because it there is internal energy lost while travelling inside inside the excited state.

The excited state is not one energy state, but it is pretty thick containing large number of very closely packed energy states though they are very close to one another, but the energy difference between them are very small, but finite and if you had passed through several of them you do lose some amount of energy and thereby the emitted light the emitted light

is of a lower frequency lower energy higher wavelength than the input light input photon. Now, in the very first lecture of this particular module we have discussed about endogenous and exogenous ah fluorophores. Several of biological compound fluoresce by themselves meaning if you excite the electron in these molecules these materials present in biological matter they will have ah they will emit they will emit back light of a lower frequency. I even give you examples flavins as in vitamin B 2 riboflavin etcetera nicotinamide adenine dinucleotide and then elastin and collagen. So, several of the biological matters ah these are NADPH as I said are enzymes elastin and collagen are part of your muscles and proteins type things they help glue or bound together.

So, they could very well be detected using ah just shining high frequency light on them, but if you want to look for other things such as DNA some specific protein they do not exhibit fluorescence. So, you cannot image DNA just by shining high frequency light at them at least they will not emit reflection you might get into trouble because of diffraction limit. Diffraction limit determines whether the light will be able to resolve it or not, but it can still be utilized for fluorescence to emit ah a different wavelength of light right and these wavelengths are usually at a lower frequency right.



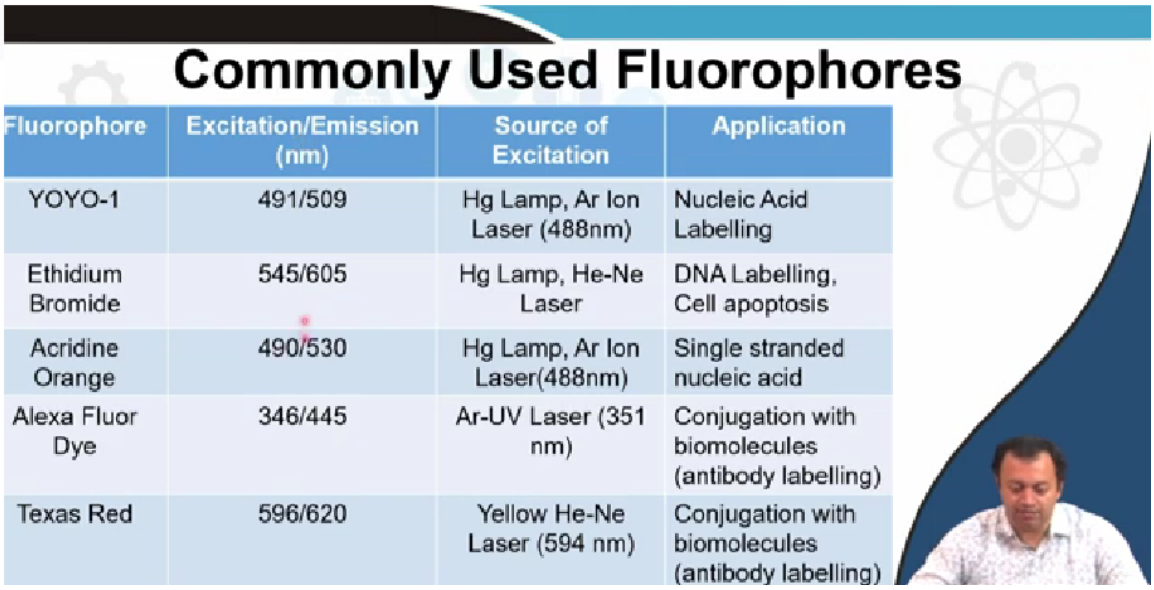
So, thereby we go for exogenous fluorophores exogenous fluorophores are those artificial compounds artificial materials that are made to attach that are made to fix that are made to glue with a specific specific part of a particular ah biological matter. It could be cell membrane it could be some some some specific area of the cell mitochondria or nucleus some part of protein and it is like labeling just like using your highlighter your sketch pen you label a particular area of your textbook.

The textbook contains thousands of lines thousands of pages thousands of you know

paragraphs which is the most important one the one which you want to study you want to memorize you want to understand you want to look up all the time you highlight it you highlight it using a highlighter using a sketch pen this is exactly what we do in fluorescence we attach it we label it hence it is called labeling with a target molecule. The target molecule is that important passage important line on your textbook and then you shine light at it the illuminated light or the the the emitted light illuminate that area and you can immediately understand locate ah what exactly is going on this is an example of fluorofold level human cells. Ah your exogenous fluorofolds have to have high quantum efficiency quantum efficiencies where the number of input photons is converted into number of output photons that is basically the difference how many amount of photon input has been converted into emission with some amount be getting lost as as as non radiative transition. It has to be environmental stable and absence of photo bleaching photo bleaching is degradation it is basically degradation an expiry date like your ah medicines expire after certain time exogenous fluorofold stop emitting light after a certain period of time right their chemical composition degrades their chemical processes changes through same like medicine same like medicine it simply accept light from somewhere else the electron moves for a longer period of time for a longer period of time ah it can photo bleach. Exogenous fluorofolds we need to create some artificial fluorofolds artificial materials that accept light and so fluoresce produce fluorescence effect are called fluorofolds that need to have high environmental ah sorry high quantum efficiency and they had to be environmentally stable and safe right you cannot put very toxic ah these kinds of chemicals inside a cell and then want to monitor the cell while the presence of these these toxic artificial materials are killing the cell itself then it it it it a a it defeats the purpose.

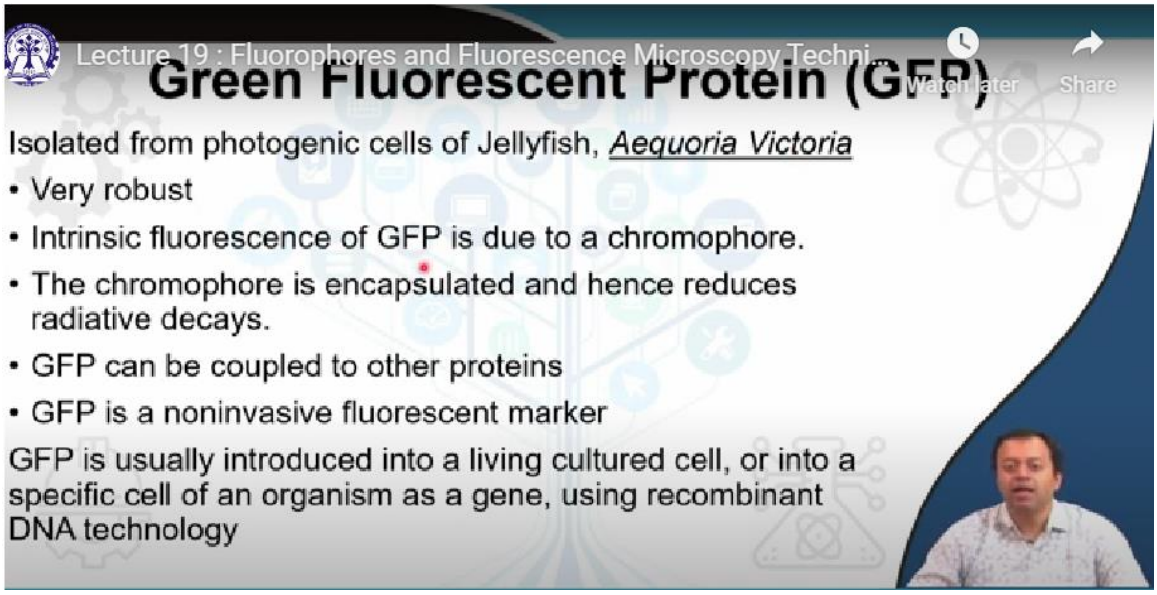
So, ah when exogenous fluorophores are therefore, quite important and quite sophisticated. So, fluorescence labeling bio imaging of a specific organelle or a site in a cell can be accomplished to study its structure and function as I said it will illuminate these

fluorescence probes can be divided into fluorophore targeting biological molecules anything organelle is without any prior coupling to a biomolecule or fluorophores that need to be conjugated to a biomolecule in order to acquire specificity for a certain biological site. One is more or less direct it does not require any coupling beforehand another is simply where you couple a fluorophore that needs to be connected with a biomolecule at a specific biological site you need a medium you need a medium for these fluorophores like antibody is connected with fluorophore and that antibody is connected to an antigen and then you are understanding then you are illuminating or fluorophores that will simply be sitting on to some organelle is directly directly this is direct this is indirect this require a medium like an antibody this can directly stick with ah a biomolecule that you that you using hydrophobic hydrophilic interaction electrostatic even and then you image it. Here you require an intermediary to connect it with a specific agent specific portion of the ah biological cell here you require nothing it can directly sits with what you want to see here you do not want to image antibody you want to image what antibody is connected with here you want to image exactly ah the biomolecule that you want to see. These are some of the commercially available fluorophore I have used YOYO-1.

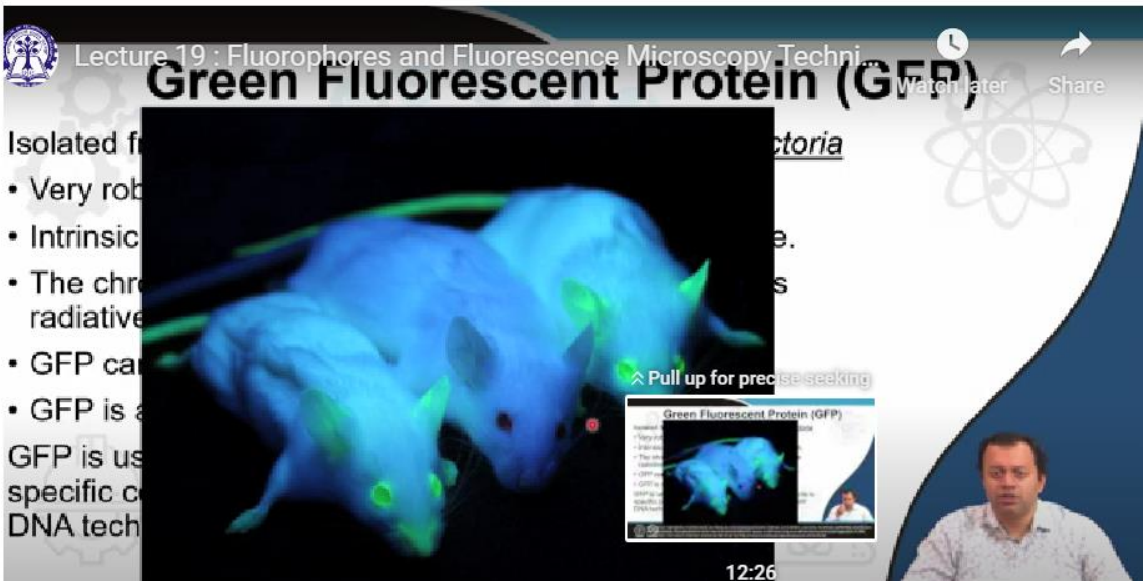


So, these are the excitation emission these YOYO-1 will be connecting themselves with certain biomolecules inside the cell you shine light on to them a laser light of 491 to 500 nanometer and ah sorry 491 nanometer and they will excite at 509 nanometers. So, 545, 605 so visible ah green to visible red. So, excitation versus emission ah things source of excitation is a laser or just a mercury lamp and here is a chart of where they are utilized YOYO can be utilized with nucleic acid DNA ah acridine orange can be for single nucleic acid conjugated biomolecules etcetera all of those things these are the company name these are the brand name of those exogenous fluorophores. And one of the most important one the person got Nobel Prize for discovering this is the so called GFP Green Fluorescence Protein this is a amazing fluorescence material is an amazing fluorophore it is isolated

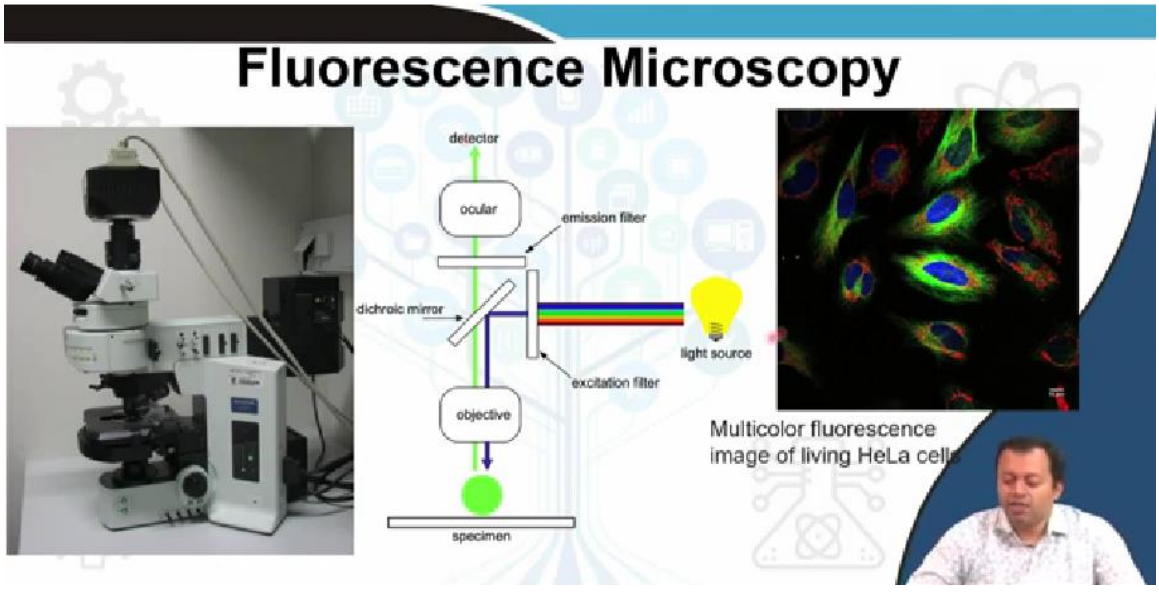
from a biological ah fish some kind of a jellyfish aquaria victoria it is very very robust and it produces upon excitement by blue light a green light. Most important thing why why GFP is not like these fluorophores these are artificially created and they are made to attach with a specific specific biomolecule and then you see it.



GFP on the other hand can be utilized to be produced by a cell itself understand this. I told you in the biology class that DNA produces RNA RNA produces proteins GFP is a protein in itself if you are clever enough if you are if you know how to manipulate DNA all you need are four bases right these four bases produces a specific protein somehow you have manipulated the DNA to produce GFP so jellyfish produces this protein anyways somehow you have added that that DNA of the jellyfish that produces GFP into another organism into another organism using recombinant DNA technology. This technology is pretty sharp and people have been using it few days ago a few years ago they got Nobel Prize in CRISPR which basically is a technology in which you can manipulate the DNA manipulate the gene. So, GFP is usually introduced into a living cultured cell or into a specific cell of an organism and the organism will live organism will breathe it is just it is producing a particular protein that fluoresce in itself. Let me give you an example this is an example the jellyfish's DNA have been incorporated into these mouses specific areas of these mouses and when you put them in blue light they fluoresce from certain area they fluoresce from certain area by adding the DNA of jellyfish inside these mouses.

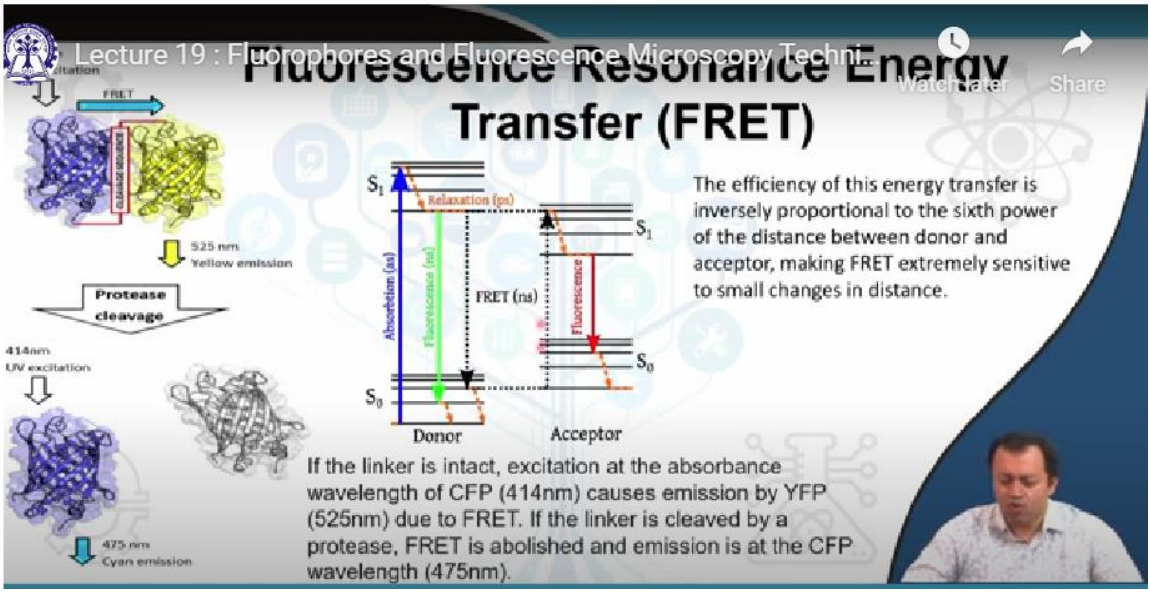


Would you like to have that in your body think about how people will be amazed if a part of your body illuminate when you go inside a discotheque where blue light is present right. This is possible and here say we want to monitor or understand the eyes of this mouse how it is forming the retina etcetera we will simply need to put it in the you know blue light and some microscope and we will be seeing the emission will be there to see this is the control normal mouse versus a mouse whose DNA is producing green fluorescence protein. Jellyfish probably you have seen jellyfish glows in dark bioluminescence and what not is the same thing DNA from jellyfish is injected inside human body. Now they are thinking of injecting it inside sorry mouse body inside human body to as a fluorescence tattoo some kind of a label which otherwise is invisible to the naked eye, but once you once you illuminate it using ah some sort of a blue light torch you can see that right.



So, fluorescence microscopy is therefore, have been revolutionizing you have a light source that is filtered out a particular blue light is made to fall on a particular specimen that specimen contains fluorophore either directly connected with the biological material or it is connected via something blue light is the input the electron goes up goes through some sort of a transition returns back to the original place by emitting green light.

This green light you can put beam splitter you can put emission filter you can put pin hole measurement you can put by this focus that focus and comes into your detector. Result is beautiful beautiful images of HeLa cell ah cancerous cells where multiple fluorophores are made to attach to multiple areas. So, one fluorophore say YOYO1 is made to attach with the nucleus then the other one is made to attach with cell membrane and thereby or all these red blue color one at a time is filtered out these filters all are dynamic they are not specific all they are dynamic and then you put it into a computer and they scan it laser scanning confocal microscope or laser scanning confocal fluorescence microscope are very very popular in most biological labs biochemistry labs and they label it regularly and thereby image ah beautifully beautifully cells. It is one thing to image cell which are simply reflecting yeah and then there is one thing to image cells which are emitting light emission is an active process it will always always be brighter no matter how how polished your surface is your light source will still be brighter yeah polished surface is simply reflecting a bulb is is is on the other hand producing creating light.

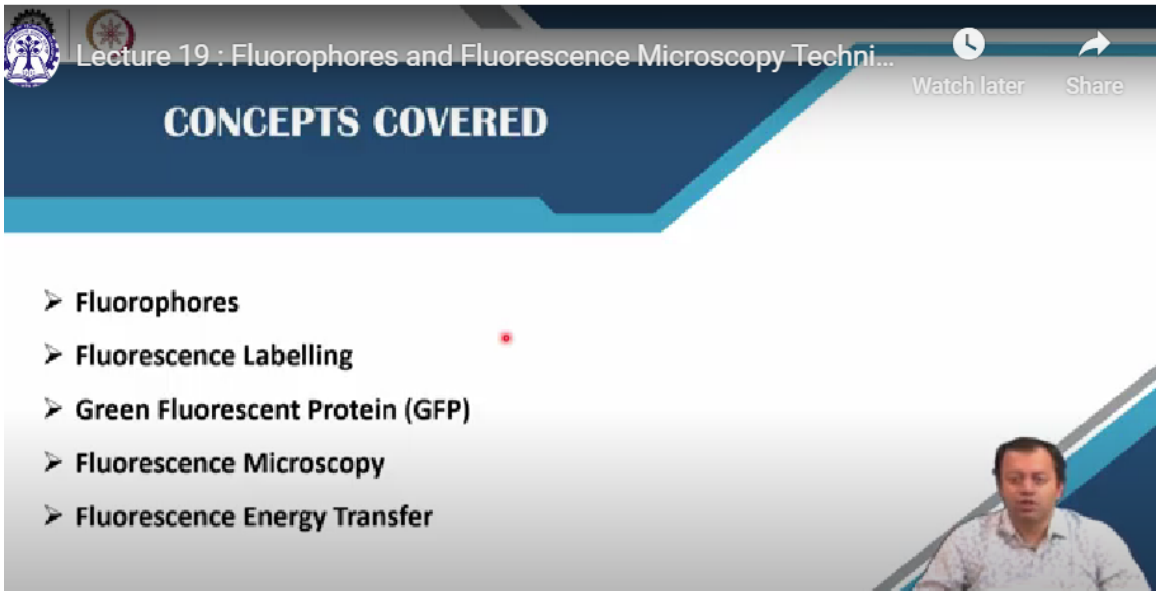


So, there is also this thing called ah fluorescence resonance energy transfer in which you have two different molecules a donor and an acceptor the output of a donor.

So, whatever fluorescence the donor is ah sending out ah acceptor is another fluorophore. So, the output of this donor can be considered as an acceptor of this. So, there are two

fluorophores molecule A molecule B both of them fluoresce coincidentally the output emission of donor matches that of the input donation of the acceptor. So, in a solution if you have two different types of ah proteins or two different types of materials one is labeled with fluorophore A one is labeled with fluorophore B donor and acceptor they are far apart from one another they are far apart from one another you have excited this with blue light you have excited this will green light this is emitting blue green this is emitting red life is good life is fine, but you have started bringing them closer to one another closer to one another when they are very very close to one another then there is a energy transfer resonance energy transfer. So, whatever donor is releasing is getting accepted is immediately getting absorbed by the acceptor and the output is only this red light no green light is getting emitted.

So, that determines the overall biochemical reaction how far or how close how far or how close these two proteins are they are very very important to know docking system how a particular virus is attaching itself to the cell how close it needs to come what exactly is happening previously in your screen live imaging you were seeing green light and red light green light and red light now suddenly you are seen no green light green light has suddenly extinguishes if someone has switched it off and you have only red light coming up. So, ah FRET, Forster resonance energy transfer or fluorescence resonance energy transfers help us in understanding several biochemical image informations biochemical reactions several immunological responses all of those things can be detected using fluorescence resonance energy transfer mechanism.



So, these are more or less the concepts covered by today for me and these are my references. So, I will see you in the next class. Thank you.