Molecular Biology
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Module - 02
Basics of Biological system (Part 2)
Lecture-10 Program Cell Death

Hello everyone! This is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering IIT Guwahati and what we were discussing we were discussing about the different aspects of the cells or the biological system. In this context so far what we have discussed we have discussed about the structure of the cells whether it is a prokaryotic cell or the eukaryotic cell. So in the current module what we are discussing we are discussing about the cellular metabolism so we have discussed about the carbohydrate metabolisms, lipid metabolisms and in addition when we have also discussed about the protein synthesis and so all the purpose of these catabolic reaction is to generate the energy and then this energy is actually going to be utilized for the synthesis of the new biomolecules and if you see that the synthesis of these new biomolecules is directly linked to the growth of that particular organism and subsequent to that it is actually going to result into the division of these cells and so that the number of cells are actually going to increase and this is what we have discussed in the previous lecture. In the previous lecture we have discussed about the cell division and how the different phases are present in the cell division during the and we have discussed about the interface we have discussed about the S phase G1 and G2 and as well as the M phases and in the previous lecture we have also seen how you can be able to how you can be able to experimentally verify these cells in the different stages whether it is mitosis, meiosis or the different cell cycles studied by the effects flow cytometry. In the current lecture what we are going to discuss we are going to discuss about the programmed cell death.

So as the name suggests this is a this these are the set of set of the cascades of reactions which are going to be governed by the molecular players and they are responsible for the death of the cell in a very very systematic way so that it should not cause any harm to the organisms. Now first let's discuss about what is the life cycle of a cell. So if you think about the life cycle of a cell it starts with a cell okay whether it is a prokaryotic cell or the eukaryotic cell and when the cell take up the nutrition from the outside it is actually going to take this nutrition and generate the energy right this energy it is going to be generated by the catabolic reactions and we have there we have discussed about the carbohydrate metabolism and as well as the lipid metabolisms this energy is actually going to be used for many purposes such as this is going to be used for the growth of the cell and it is also going to be used for the reproduction right because every cell wants to increase its number so it's going to be used for the reproduction of the cell. Now this will

continue until the uptake of the nutrition and the production of energy would be on a higher side compared to the energy what it requires for the other kinds of processes and after some time when the cell will go through a process of aging what happen is that this cell will actually go to the different types of changes right.

One of the things one of the serious change is that it's actually going to enter into a non dividing phase which is called as G0 phase and once it enters into the G0 phase it will stop the division right so it will not going to produce new cells and on the other hand it is actually going to just maintain the basal level of activities and as a result it is not going to perform many functions. After this it will enter into another phase which is called as death phase right because the every every cell has its definite lifespan so it is actually going to enter into the death and the death within the cell can be induced by the two different processes. It can be done either by a programmed method or a programmed manner which is called as programmed cell death or apoptosis or it can be done in a another method which is called as necrosis okay. We are going to discuss in detail about the differences between the Bogram cell death apoptosis as well as the necrosis and what are the contrasting feature of these two things so and either of these methods are responsible for the death of the cell right and the cell has to take a decision whether it wants to go for the apoptotic pathway or the necrotic pathway. So in today's lecture what we are going to discuss we are going to discuss what actually induces the cell to go for a suicide pathway or apoptotic pathway.

What is the definition of the apoptosis and its major features right so what are the different hallmarks of the apoptosis what are the different events are happening and then the steps involved in the apoptosis difference between the apoptosis and the necrosis the pathway which are involved in the apoptosis so apoptosis can be produced or can be induced by the external factors and as well as the internal factors and then what is the relevance of the apoptosis in the overall biology of the organisms how it is actually affecting the other kinds of processes especially the development. Now the first question comes why the cell actually commits the suicide or I will say program death okay. So as we discussed right the cell is actually going to take up the nutrition and this nutrition is actually going to produce the energy in the form of the ATP or energy in the form of reducing equivalents such as NADH right and the purpose of these energy sources are that it is actually going to be used for the growth of the cell it is actually going to be used for reproduction and apart from that it is also going to be used for maintaining the cellular integrity okay. What is mean by the cellular integrity is that this energy is actually going to be utilized for maintaining the electrode potential or plasma membrane potential right. So actually what happened is that it is actually going to run the pumps right sodium potassium pumps or proton pumps and as a result it is actually going to throw the proton outside right and so that there will be a potential what is going to be

developed right.

So it is actually going to generate a potential across the plasma membrane because of that it is actually going to have the negative charge inside and positive charge outside or I will say it is actually going to have the negative polarity onto the plasma membrane and because of that if you have any object which is outside like for example the glucose molecule right. So glucose will not enter into the cell because it does not it is actually a charged membrane so this molecule cannot enter passively inside the cell it has to go through by a process of a receptor. So it actually has to go through with a either through the receptor mediated endocytosis or it actually has to use the transporters to enter into the cell right and that's how it actually mean going to decide or it is actually going to maintain a electrode potential and that electrode potential is helpful in terms of stopping the entry of the external factors. For example if you have a water molecule right the water molecule can easily enter into the cell right but it does not because the water is will actually going to use the water channels or water is actually going to be because if you can imagine that if the water will keep coming into the cell the cell will actually going to expand right so it is actually going to increase its size and ultimately it is actually going to burst right and that is what is going to happen when you are actually going to have the osmotic lysis. So these are the some of the things which actually cell opposes throughout its life right and for that only it is actually spending the energy in the form of the ATP or NADH.

Apart from that it also require the energy for any many more things actually it's okay. So once the cell is weak or cell is getting through a process of aging it actually has reduced its ability to produce the enough amount of energy and when it does not produce the enough amount of energy what it actually can do is it is actually has to cut down the activities it has to cut down the activities so it actually will cut down the active first thing what it will actually going to do is it will cut down the activity of cell disease right this means it will enter into the G0 phase just to conserve the energy right it will enter into the G0 phase. In the second event what it actually will do is it will actually going to you know it will not going to use the you know so if if the energy is still going to be on a lower side then it will decide whether I should be able to maintain my integrity or not because cell is cell only until it is actually having a integrate system actually okay. Once it actually become porous right and it can allow the cells allow the entry of the external molecules then the cell will eventually going to burst and since the cell will actually going to burst and release its content into the external media it is not going to be good for the organism because if you release the cellular content in one shot right it is actually going to create the you know the disturbance to the homostasis. So because to avoid this what it actually going to do is when it reaches to that point where it will not be able to maintain or it will not be able to generate the enough energy what it actually going to do

is it is actually going to induce the programmed cell death which means it was actually going to say that okay I am no longer be in a state that I can be able to maintain my integrity so let us go for the death pathway and then only it is actually going to go with the death and as I said you know death could be induced by the apoptosis or it could be necrosis.

So apoptosis or the cell this programmed cell death is a fundamental process in a multicellular organism which play a crucial role in the various biological processes such as development, tissue homostasis, immune response and the elimination of the damage or the potentially harmful cell. So this is you don't have to worry about all this because this is all we are going to discuss at the end of this lecture how the apoptosis is involved in development, tissue homostasis and elimination of the potentially harmful cells and all that. Apoptosis is a tightly regulated physiological process that rapidly removes damaged, mutated or virus infected cell within organisms and the major feature of an apoptosis that it is a controlled and the ordered process in contrast to the necrosis. Okay so apoptosis is also called as programmed cell death which means you are actually going to do the programming to have the death of that particular cell right that say it is actually going to be a controlled and ordered process because you are going to do a programming I'm sure many of you probably know about programming right so you actually gives the steps or you are going to give the command to the particular computer right okay go with this go with this go with this right and ultimately execute this and do this job right so the same way you are actually going to go with the stepwise instruction to the cell and eventually the last instruction would be that okay induce the death right then we have the specific signaling pathway and the molecular event which actually going to drive the process and this all how you are going to do you are actually going to do this by having the specific signaling pathways because these signaling pathways are actually going to bring the molecular players and that's how they are actually going to make the process more controlled then we cellular components are dismantled leading to the cell shrinkage then DNA fragmentation occurs resulting into the characteristic ladder pattern apoptotic bodies are apoptotic bodies small membrane bound vesicles which are actually going to be formed so shrinkage of the cell cinderella ring and as well as the formation of the apoptotic body these are the hallmarks of the apoptosis so if I have to identify an apoptosis in a cell I will see whether it is forming the DNA fragmentation or not if it whether there are apoptosomes are formed or not and whether the cytosol is shrinking or not right so that is are very different from the from the necrosis now what are the steps are involved in apoptosis so apoptosis actually is going to start from the cell right so its first step would be that the cell is actually going to receive a signal right this signal could be from the external signal or it could be an internal signal okay so it actually first step is that it actually going to receive the stimulus this is stimulus would be external stimulus or it could be an internal stimulus then once they receive the stimulus it actually going to

induce the shrinkage as well as the breakdown of the cytoskeleton which means it's actually going to make the cell more flexible so that it can actually be able to get condensed then we have the dense cytoplasm and the packaging of the organelles then we have the condensation of the chromatin this process is called as the pykniosis then we have the induction of the caspase activated dns activity and that will induce the fragmentation of the DNA means this is the fragmentation of the genomic DNA and breaking of the nucleus the step 6 it is actually going to form the blobs and then step 7 there will be a cell break and the formation of the apoptotic bodies so these are the formation of the apoptotic bodies this means the individual cellular content is actually going to be encircled into the plasma membrane and that is actually going to be apoptotic bodies these apoptotic bodies are not going to release the content they are actually going to be taken up by the macrophages and ultimately is that it is actually going to be phagocytose so that's will be that's why this is actually going to be a very safe way of removing the particular dead or damaged tissue without even causing any kind of adverse reactions now what is the difference between the between the apoptosis and necrosis so in the apoptosis you are going to have the intact cell membrane and membrane blubbing is going to occur right so this is actually going to happen right so it is actually going to maintain intact cell membrane but there will be a blubbing into the membrane okay compared to that in the necrosis there will be a disrupted cell membrane and there will be a loss of membrane integrity right so this there will be a loss of membrane integrity this means it is actually going to start losing the cellular content as soon as the necrosis is going to occur into the cell right and in the step 2 it's begin with the cell shrinkage and the condensation of the nucleus and then the picnosis occurs which means the condensation of the chromatid and followed by the karyohexesis which is the fragmentation of the nucleus and ultimately there will be a formation of the apoptotic bodies so these are the formation of apoptotic bodies which means it is actually not going to release the cellular content into the external media or the outside instead it is actually going to release the apoptotic bodies and these apoptotic bodies are actually going to be psychocytosed by the macrophages so that they will be very very very safely they are actually going to be removed from the from the environment compared to that the in the during the necrosis it is actually going to be it begins with the swelling of the cell right so initially the cell is actually going to swell because it cannot maintain the integrity right followed by the picnosis and the karyohexesis occurs followed by the karyolysis which means there will be a dissolution of the cytoplasm and ultimately there will be a complete lysis of the cell which will and there will be no apoptophome which is going to be formed and then ultimately it is actually going to release the complete cellular content and as a result of that it is actually going to cause a huge inflammatory reaction because this cellular content is going to be you know it actually going to attract the many type of cellular many type of immune cells and once they come to that they will actually going to try to clear this and they will actually going to cause the

inflammatory reaction which means they are actually going to secrete the inflammatory molecules and as a result there will be more damage into the vicinity where the necrosis is going to occur. Now coming back to the apoptosis there are multiple pathways which are involved into the apoptosis okay so in the apoptosis you have the three steps first is initiation second is execution the third is the psychocytosis okay because after the execution it is actually going to form the apoptosome and or apoptotic bodies which are and these apoptosomes are actually going to be psychocytosed okay so this two and step number two and three are actually going to be the same for both the pathway but the initiation pathway can be different for the two pathway okay because the first step is the stimulus right so if the stimulus is internal right then it is going to be intrinsic pathway if the stimulus is external then it is going to be intrinsic pathway or extrinsic pathway so that is the only difference okay if it is a internal factors such as starvation loss of nutrition generation of free radicals and all other kind of things then it is going to be induced the intrinsic pathway if it is the extrinsic pathway then it could be external factors such as the immunological molecules or the external ROS or drug molecules and so on okay so if you have these then it is actually going to induce that external factors it is going to cause an intrinsic pathway extrinsic pathway whereas for intrinsic pathway it could be the starvation it could be the ROS internal ROS or it could be any other kinds of anomalies that actually going to be responsible for the intrinsic pathway once the initiation is done then it is actually going to have the execution so in the execution you are going to have the caspases different types of caspases and then ultimately it is going to form the apoptosome and that actually is going to be the phagocytosed by the phagosomes so let's first discuss about the intrinsic pathway so in the mammals the signals that induce the apoptosis can either originate from the inside of the cell that is the intrinsic pathway or from the outside of cell which is called as the extrinsic pathway both signaling cascade ultimately leads to the caspase activation which in many define the number of in define the point of no return for the cell death these dead signaling at the events appear to be funneled to the mitochondria before the execution of the death by caspases into the mammalian cells so what are the molecular players involved into the apoproteins pathways first is caspases so caspases are the proteases are caspases are the group of protein involved into the apoproteins process they are called they are so called because they contain a key cysteine residue into the catalytic site and selectively cleave the protein at a site and selectively cleave protein at a site just C-terminal to the aspartate residue and caspases are the proteases all caspases are initially made as the pro caspases which means they are actually going to be produced as the inactive protease and ultimately they are actually going to be you know generate the active protease and by doing so they are actually going to be under the fine control which was cell will contain the inactive protease but it will actually going when it's going to get the signal that okay convert the inactive protease to the active protease it is actually going to be start the cascade of reactions then we also have the pro apoptotic factors and as well as the anti

apoptotic factors so within the pro apoptotic factor these factors are promoting the apoptosis such as bags bag with box bits back etc whereas we have the anti apoptotic factors these factors are inhibiting the apoptosis this means they are actually promoting the growth so these are called BCL 2 BCL-XL MCL 1a etc and the ratio between the anti apoptotic BCL 2 and the pro apoptotic batch protein determine whether a cell will actually going to live or it is actually going to die with by the process of apoptosis.

Now in the intrinsic pathway or the mitochondrial pathway you are going to have the you're going to have the initial signal right so there will be a signal which is like DNA lessons for example if you have generated the mutations or DNA damage and these DNA damage are beyond the repairing okay because some of the DNA damage can be could be repaired but some of the DNA damage could be so much that it will decide that okay it is not worthwhile to you know to to repair the damage so instead of that it is actually going to in take up that as a initiation signal and then it is actually going to activate the serine and the atm cm kinase and that in turn is actually going to activate the production of the p53 transcription factors and the p53 is actually going to activate the downstream molecules like puma then puma is actually going to activate the BACs within the cytosol and the BACs activated BACs becomes the mitochondrial membrane bound and as a result it is actually going to open the voltage-gated channels right and it actually going to form the membrane attacking complex and once that happens it is actually going to release the cytochrome C from the mitochondria into the cytosol and that is actually going to be initial event right once the cytochrome C is actually going to be released it is actually going to form a complex with the APA one right and once they will form a complex to meet each other it is actually going to form the apoprosome which means the APA one and the cytochrome C when they come together they are actually going to form the apoprosome and apoprosome is actually going to activate or it's going to recruit the inactive caspase 9 and it is actually going to be activated to form the active caspase 9 so from the active caspase 9 it is actually going to act on to the pro caspase so it's actually going to form the pro caspase 3 and it's actually going to act on the pro caspase 3 okay and then it becomes activated caspase 3 so these are called executory caspase these are called initiator caspase so activated caspase 3 is going to form an activated caspase 3 is actually going to activate the caspase activated the DNA's the once the caspase activated DNA is formed it will actually go inside the nucleus and then it is actually going to form the it is actually going to start chewing the DNA but it is very specific so it is actually going to chew the DNA after every 180 nucleotides okay and that's how it is actually going to form a ladder like things right because it's every ladder is actually going to be different from each other by a number of 180 so first DNA is going to be 180 first second DNA is 360 then 540 then 720 like that okay so that's why it is actually going to form a ladder like this okay where you are in between the two DNA is back bands it is actually going to have a difference of 180 base pairs okay this

all you will understand when we are going to talk about the DNA packaging so it will understand why there is a difference of 180 base pair right and apart from the caspase activated DNA's the caspase 3 is also going to be start acting on to the cytosolic as well as a nuclear protein and it is also going to start chewing up those proteins so as a result it is actually going to disturb the cellular machinery and at the end it is actually going to cause the cell death then we have the extrinsic pathways extrinsic pathway could be of two types extrinsic pathway where you have the TNF pathway or the fast like fast pathway in a TNF pathway you are actually going is going to be activated by the TNF alpha which is going to be secreted by the macrophages and other immune cells whereas fast pathway is actually going to be activated by the fast ligand and fast ligand is present on to the some of the immune systems or immune cells so first discuss about the TNF pathway so in a TNF pathway the acceptor TNR1 so this is actually going to be the receptor what is being responsible for the TNF pathway so on one side it is actually going to have the so this is an inactivated TNF receptor 1 and why it is in the inactive because it actually contains the death domain which is going to be captured right and on the top it does not have the TNF alpha right so it is receptor TNF R1 contain our intracellular part contains the death domain and this present on cell which is going to receive an apoptotic signal the death domain is silent prior to the apoptotic signal therefore it is called as inactivated TNF alpha TNF R1 the TNF R1 receives an external signal triggering molecule called TNF alpha cytokines which initiate apoptosis conformational changes occurs on to the intracellular part of death domain when the TNF alpha binds with the TNF alpha receptor 1 the death domain contains an inhibitory protein called SODD or the silence of death domain that keeps the death domain silent therefore the cell survives so what happened is that inactivated TNF alpha when it enters the TNF alpha will actually go into bind this TNF alpha can come from another micro immune cell such as macrophages and as soon as that happens that the domain is actually going to be active and then it is actually going to recruit the other cytosolic factors like thread FADD and then ultimately it is actually going to activate the caspase 8 ok so it's actually going to cleave the pro caspase 8 and that is actually going to make the active caspase and once the active caspase 8 is going to form it is actually going to induce a cell death by taking the help of the caspase 3 so after this actually it is actually going to activate the caspase 3 ok then we have the fast pathway right so in the fast pathway you have the two different types of protein one is signaling protein or I will say cytotoxic T lymphocyte for example right so example is cytotoxic lin lymphocytes where you are going to have the target protein target protein is the cell which actually going to be go through process of apoptosis right so this is the apoptotic cell ok and this protein the target protein is actually going to have the fast receptor whereas the signaling cell is actually going to have the fast ligand ok and once the fast ligand which is present on to the signaling cell is actually going to interact with the fast receptor what is present on the target cell it is actually going to induce the apoptosis and so how it happens you have the

fast ligand what is present on to the signaling what is present on to the signaling cell and then you also have the fast receptor what is present on to the target cell and when they interact with each other it is actually going to give you the signal and once they give the signal the death domain is actually going to recruit the downstream effector molecules and as a result it is actually going to activate the pro caspase 8 to form the active caspase 8 and the active caspase 8 is actually going to activate the pro caspase 3 to form the active caspase 3 and once the active caspase 3 is going to form it is going to act on to the genomic DNA and as well as the cytosolic protein and that's how it is actually going to induce a cell death so sickling cell is an immune cell which is called as cytotoxic telomephocytes the cytotoxic telomephocytes express a protein which is called as fast ligand the LIS ligand initiate the apoptosis through a series of reactions the fast ligand binds to the target cell through a fast receptor present on it binding of the fast ligand with fast receptor send the first apoptotic signal fast receptor contains the intracellular death domain on binding the dot domain recruit a FADD that the fats associated with death domain adapter molecule that comes and binds to the death domain of the fast receptor the death effector domain or DED of FADD molecule further recruit caspase 8 which gets activated and form the caspase 8 then a bunch of molecule existing together which are called as the activated fast receptor or the FADD adapter molecule DED and a caspase 8 enzyme form a single complex called the disk right or death inducing signaling complex the death caspase cascade starts when caspase 8 is released from the disk and what happened that caspase 8 is actually going to activate the caspase 3 into the caspase into the caspase active caspase 3 and the active caspase 3 is actually going to act on to the genomic DNA and as well as on to the cytosolic as well as the nuclear protein and eventually it is going to induce the cell death okay. Now what will be the what will be the relevance of these apoptotic pathway apart from the death okay it also has a relevance in many other features of the organisms. So the one of the major the area where the apoptosis has a relevance is the development right. So apoptosis is necessary in many developmental process during the limb formation the separate digits evolve by the death of the interdigential mesenchymal tissue. So what you see here is that this is the this is the two hands actually.

So in when you when the baby is within the womb right or whether during the developmental stage it actually has the hand which actually contains the membranes okay which actually contains the skin actually right and these hands are called raised hand okay and once baby is born these cells which are actually be a part of this web is actually going to be digested or which is actually going to be killed by a process which is called as programmed cell death and as a result we are actually going to have the individual heads. In the case of frog for example right frog does not have the individual fingers right it actually has the finger like this because it helps the frog to float onto the cell onto the water actually. Then we have the deletion of the cell no longer needed such

as the amphibian tadpole tail during the metamorphosis. So when the amphibian when the frog is developing from the tadpole so this is the tadpole right it contains a very long tail right but this tail is getting regressed when it is actually forming the adult frog actually what happens is this the cell what is present in this tail is actually going to be you know removed by a process which is called as the apoptosis. Then demise of the cell showing the structuring of the hollow tissue so this is this is what happening when you are actually going to form the from the body cellome right okay.

So during the development you are actually going to have the degradation of these cells or death of these cells so that's how it is actually going to form a tube like structure for example the development of the element chicken off. Then we have the formation of the reproductive organs and the massive cell death occur during early stage of nervous system greater than 50% of the cell neural cell actually are going to die. So what is the conclusion? In conclusion the apoptosis is a crucial process in a multicellular organism it is a controlled and ordered leaf form of cell death that play a vital role in development tissue processes and the immune system. Apoptosis is regulated by the specific pathway and involve the molecular event leading to the cell dismantling and the formation of apoptotic body. This regulation of apoptosis associated with the various type of issue one is one of such thing is formation of the you know formation of the fingers with the web actually.

So in some of the kids when they are born these webs are already present because the apoptosis does not occur and they are actually going to have this and in those cases what people do is they will actually going to be surgically be removed by the doctor actually or sometime what you see here is that the fingers are actually fused with each other. So in that case is also it is actually going to be surgically be removed because the apoptosis did not get induced in those particular people actually. Then understanding the mechanism and the significance of apoptosis opens venues for the septic intervention and shed light on the fundamental process of life and death. So this is all about the apoptosis. Now if you would like to study the apoptosis in a cell you can actually be able to use some of the classical features.

For example in there when you have a cell and if it is inducing if you are suppose treating this cell with a anti-cancer drug. So eventually what happened is you are actually inducing which you are so you are actually activating the in extensive pathway okay or you can actually inducing the intrinsic pathway whichever the you know because it depends on the different types of cell right. So different types of molecules so you can actually be able to have both of these. So what happened is the cell is actually going to show you the three important features which can be exploited for studying the apoptosis. One is there will be a shrinkage or cell shrinkage right.

The second is there will be a DNA damage and the third is there will be a membrane polarity and all of these methods apart from that you can also have the caspase activation. So you can actually be able to if you want to study the apoptosis you can use any either of these methods you can actually go with the DNA damage and look for the cell shrinkage you can look for the membrane polarity. Membrane polarity there will be a loss of membrane polarity there will be a loss of molecules from that particular cell. So one of the very easy thing is that you can actually be able to stain the cell with particular type of dye and the dye is actually going to show you whether there will be a cell damage membrane polarity or the caspase activation. So you can actually have the different types of substrate what you can actually use or you can use the dyes.

So one such approach is that where you are actually can use the combination of dyes which is called as a acridine ion propidium iodide and that you can actually be able to use for the monitoring the apoptosis. So what is the basic principle of this particular assay? Propidium iodide is a membrane impermeable dye that selectively bind the DNA by inter-calating into the double helix in live cell which has an intact membrane which means which actually has the intact membrane integrity. Propidium iodide is unable to enter the cell and therefore does not stain the nucleus. However in cell with the compromised membrane integrity such as dead cells, propidium iodide can penetrate the plasma membrane and bind the DNA resulting into the red fluorescence. So propidium iodide is actually going to give you the intense red fluorescence.

Okay to analyze apoptosis using acridine orange and propidium iodide a mixture of the dye is typically added to a cell suspension. In that cell suspension what happened is the live cell is actually going to appear green because the propidium sorry because the acridine orange is actually going to give you the green fluorescence when the cells are live but it going to give you it is going to give you the orange or red fluorescence when the cells are under the apoptosis. So this is for the live cell, this is for the apoptosis cell. So in a live so all the live cell is actually going to appear green. Apoptotic cells are going to appear orange or the red depending upon the amount of or the degree of fragmentations and the dead cells are actually going to appear as red right.

So these you are actually going to have three colors you are going to have green which is for the live, you're going to have the orange which is for the apoptotic cell and you are going to have the red which is for the dead cell right and you can easily be able to identify all of these colors in a technique which is called as flow cytometry and remember that we have very extensively discussed about the flow cytometry in our previous lecture so you can be able to utilize the flow cytometry for this particular type right. And these are the some of the material what you require for performing the AOPI

apoptosis assay and this is the protocol. So you have to take the 10,000 cells you are going to treat them with a with a substitutable anti-cancer compound or any other compound which you are thinking that it is actually going to induce the apoptotic cell death and then you can actually be able to follow this and it is actually going to give you the staining for the cells. So working consultation for the AO is 1 to 2 microgram whereas for the PI it is 20 to 50 microgram and it is actually going to add it to the cell 15 to 20 minutes before acquiring the data on a flow cytometer recuberance and as far as the data acquisition is concerned the after staining analyze the staining cell using a flow cytometer equipped with the appropriate filter for ocidine, orange and propidium iodide. Adjust the flow cytometer setting for the appropriate fluorescence and forward and side scatter parameters.

Run the stain cell sample on the flow cytometer collecting data for at least 50,000 events right. Analyze the acquired data using flow cytometer software plot the scatter plot with PI on the y-axis and the occludally orange on the x-axis. The first quadrant represent the healthy and the live cells and you can partition that plot into the four quadrant the second third and fourth quadrant represent the early apoptotic, late apoptotic and dead cell respectively and you can do the data analysis with the help of a software which is called FCS-5. You can use any other software this software is not exclusive okay and ultimately what you are going to see you're going to see the results okay. So, what you are going to see is this is the control right and this is the treat example okay.

But before discussing about the results we can actually take you to my lab and where the students are actually going to show you the complete protocol and how you can be able to analyze or do the acquisition of these data into the flow cytometer and how you can be able to analyze the data to get this result. Hello everyone, in this video we will be discussing about how to perform live dead cell staining using adiridin-orange and propridium iodide on FaxA. So the basic principle is that the adiridin-orange is permeable to both the live and dead cells whereas the propridium iodide is only permeable to late apoptotic and necrotic cells. So this property of adiridin-orange and propridium iodide lets us to recognize what population of cells are in late apoptotic or early apoptotic or necrotic cells. So coming to the procedure the first thing we do is that we treat the cells from the 100 mm cell culture dish and then plate 1 million each in the untreated and the treated well.

So after 2 to 14 hours of adherence we treat the samples according to our requirement and then let's say for that we are treating for 24 to 48 hours then after the appropriate time we crystallize the cells collect the pellet wash it 2 times with PBS and then resuspend the pellet in 2% beta 2 1 serum in phosphate percolate. So after we have resuspended the pellet in 2% and in phosphate percolate we give the appropriate

adiridin-orange and propridium iodide treatment. The working concentration for adiridin-orange is 0.5 to 1 microgram per ml whereas the working concentration for propridium iodide is 1 to 5 microgram per ml. So we add the dyes just before taking the data or we can just give 10 to 15 minutes of incubation for the dyes to bind to the cells so that and after that we record the data on the fat cell content.

So after adding the adiridin-orange and propridium iodide to the cells we have to acquire the data on the CellQuest Pro software. So the first thing we do is open the CellQuest Pro and connect it to cytometer and then we need the counters the detector and amps and the status. For the adiridin-orange, propridium iodide staining we need two dot plots. One is for the FSC, SSC for the forward scattering and the side scattering and the other one is for the FL1 and FL3. So the FL1 plot is on the x-axis whereas the FL3 plot is on the y-axis.

The FL1 plot is for the acridin-orange and FL3 plot is for the propridium iodide. After taking the plots we have to set the directory and save the data in our required location. In the detector and amps we have to remember that we have to set the population of the healthy cells in the first quadrant that is 10 to the power of 1 and 10 to the power of 1. So after we set the untreated cells in the first quadrant and then we analyze the treated cells and then we can say whether there is any shift in the fluorescence in the untreated and the treated cells. For the treated cells in the third and the fourth quadrant that represents the apoptotic and the necrotic cells.

So now we will be taking the sample but before analyzing the data we have to set the number of events to the 5000 and then keep it on setup and first we will see whether the events are coming properly or not. Now we press acquire. As we can see that in the FSC and SSC plot we can see the events coming near 0 0 that represents the healthy population as well as in the FL1 and FL3 we have set the healthy population between the 10 to the power of 1 and 10 to the power of 1. So this represents the first quadrant. We will show in detail how to do the quadrant analysis in the FCSX-5 software.

Now that we have set the population in the first quadrant we will remove it from setup and acquire the data. After the untreated samples we have to take the treated samples on the same parameter description which we have set for the untreated cells. Now we change the sample in the sample injection port to the treated sample. We have to remember that we don't have to change the parameters or else we will never be able to say whether there is any shift in the untreated or the treated cells if we change the parameters. After changing the sample now we have to choose the directory for the treated cells and then change the name also to treated and also the file count to 1.

Then press OK and then now we acquire the data on the same parameters. As we can see that there is some shift in the population of the cells. The population is having a little bit more fluorescence than untreated cells which represents the apoptotic and necrotic cells in the third and the fourth quadrant. In the fourth quadrant mostly the necrotic and the dead cells are present whereas in the third quadrant the late apoptotic cells are present. After we take the untreated and the treated samples after we acquired the data for the untreated and the treated samples we have to analyze the data in the FCS5XPress Pro software using quadrant analysis.

In the quadrant analysis we can see how much populations of cells are present in which quadrant and therefore we can identify the number of healthy populations and the apoptotic and the necrotic cells. So after acquiring the data in the FACTS equipment we have to now analyze the data in the FCS5XPress software. So the first thing we do is we open the new layout and change the orientation to landscape and then now we input the data and then first thing we do is we take the untreated file and then open the dot dot. We need two dot plots the one is the FSC SSC and the other one is the FL1 FL3. The FL1 FL3 dot dot shows the live and dead cells staining.

The FL1 is responsible for the acridin orange whereas FL3 is responsible for the propidium iodide and now we take the treated file and again we select the dot plot. In dot plot as well we need the FSC and the FL1 FL3 plot. As we can see that there is a difference between the untreated and the treated sample. Now we have to find out how much percentage of the cells have gone actually the apoptotic or necrotic. Like we have to divide the population of cells into four quadrants using the quadrants option.

So we go to the gating and then take the quadrant and then apply it on the FL1 FL3 plot. We have to apply it in such a way that we cover all the cells in the untreated plot. So let's say that in the untreated plot we are having 93 92 percent on live cells and 7 percent are in the early apoptotic phase and then after applying the quadrant to the untreated we have to apply the same quadrant to the treated one in order to find out the difference between the two. So just we click on the quadrant and then we copy and paste on the treated one. So in this way we can say that in the first quadrant in the untreated sample we have approximately 93 percent of live cells whereas in the treated one we have like in the treated one we have only 35.

7 percent healthy whereas the 33 percent have gone are in the late apoptotic and 29 percent are necrotic cells. So in this way we can use acridine orange and propidium iodide to determine the healthy the apoptotic and the necrotic cells built in different treatments and also we can establish a relation between different concentrations of treatment and the number of live and dead cells in any experiment. So this is the way we

analyze the we analyze and process the data on the FACTS equipment in order to do the live and dead cell training. Hopefully this video was helpful. So once you analyze the data you are actually going to get this data right you are going to get these two curve right or these two plots and these are called as the dot plots okay and where you are going to have the checkerboard analysis.

So this is called as checkerboard analysis right and in the checkerboard analysis what you're going to do is you're going to make a checkerboard in such a way that you are going to keep all the healthy cells in the first quadrant. So this is a quadrant 1, this is the quadrant 2, this is the quadrant 3 and this is the quadrant 4 okay. In the quadrant 1 you are going to have the low fluorescence for the FL3 and low fluorescence for the FL1 which means it is actually going to be the healthy cells because they are not taking up the strong fluorescence signal or they are not taking up the dye from the they are not taking up the dye which means they are their membrane potential, membrane polarity is very high and that's why they are not allowing the dye to enter right. When you treat these cells the cells are actually going to be apoptotic and as well as the dead so they will actually enter into the next quadrants. So for example this is the quadrant number 1 which is the healthy cells right this is the quadrant number 2 which is actually the early apoptotic cell which means now just the DNA damage started actually so and that's why what you see here is that it has a very high signal for FL1 but it has a very low signal for FL3 right this means these are early apoptotic and this is the late apoptotic because now the DNA is compromised and so it actually has a very high signal for FL1 and also has a high signal for FL3 and that's why these are the late apoptotic cells and this is the cell where you have the low fluorescence like low FL1 and high FL3 and these are the dead cells right.

So what you see here is that in the treatment you have the 27% dead cells 36% late apoptotic cells and the 11.8% early apoptotic cells and whereas 23.5% healthy cells. So these are all about the apoptosis. Now when you are doing these kind of essays you always have to take a lot of precautions when you are analyzing the data when you are making a checkerboard and all that so that you should not make mistakes so that it should be make you biased or you should not be able to get the you know the data which is unreliable.

So these are the some of the precautions what you have to follow you have to acquire the samples under the 4 degree and you also have to require other kinds of treatments other kinds of precautions what you are supposed to take. So this is all about the apoptosis or the programmed cell death what we have discussed we have discussed about why the cell is entering into the apoptosis what are the critical factors which are inducing the apoptosis and what are the different types of pathways which are responsible for

apoptosis. So we have discussed about the intrinsic pathway and we have also discussed about the extrinsic pathway within the intrinsic pathway we have discussed about the molecular players which are governing the intrinsic or the extrinsic pathways so and at the end we have also discussed about how you can be able to study the apoptosis with the help of the slow cytometry. So with this I would like to conclude my lecture here in a subsequent lecture we are going to discuss some more aspects related to molecular biology. Thank you. you