Biomaterials for Bone Tissue Engineering Applications
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Module 5
Lecture No 24

So we will continue the discussion on the differentiation assay. So the assay that I finished in the last module is that alkaline phosphatease assay.

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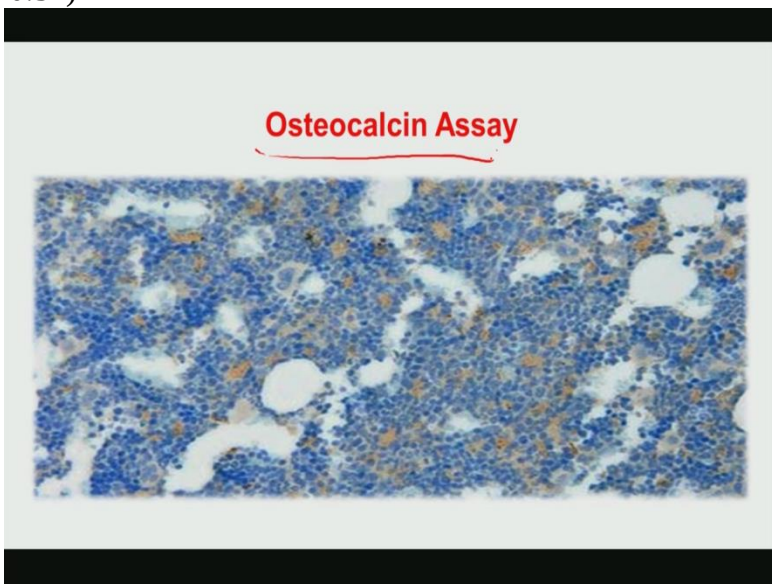

Now we will start with this late differentiation mark that is Osteocalcin. So this Osteocalcin assay is typically expressed in the later stage of differentiation.

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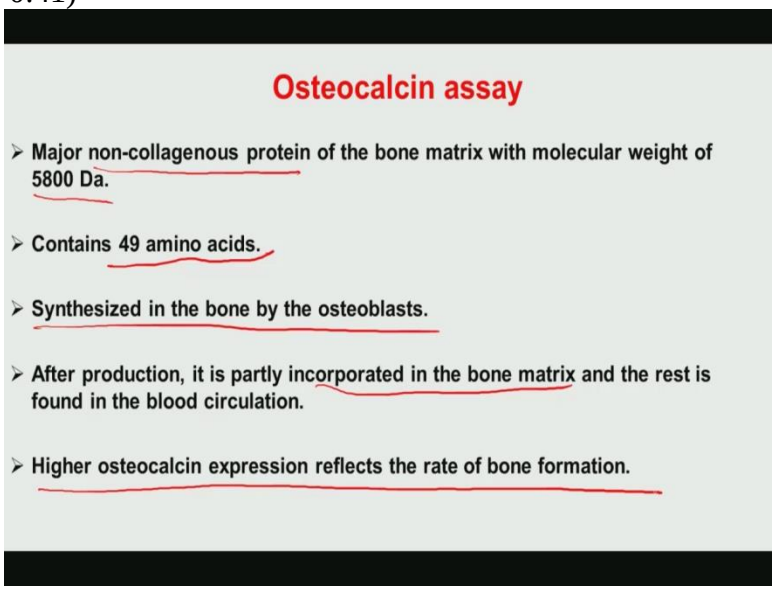

And this is just a brief background on this Osteocalcin. It is essentially non collagenous protein which has a molecular weight roughly around 5.8 kilo dalton. And it contains 49 amino acids is synthesised in bone by the osteoblasts. So essentially whenever your osteocalcin expression is up regulated or whether whenever osteocalcin expression is increased that means that that is also the reflection of the osteoblast activity since osteocalcin is synthesised in bone by the osteoblast.

Then after production it is essentially incorporated in the bone matrix and the rest is found in blood circulation. Higher osteocalcin expression reflects the rate of bone formation.

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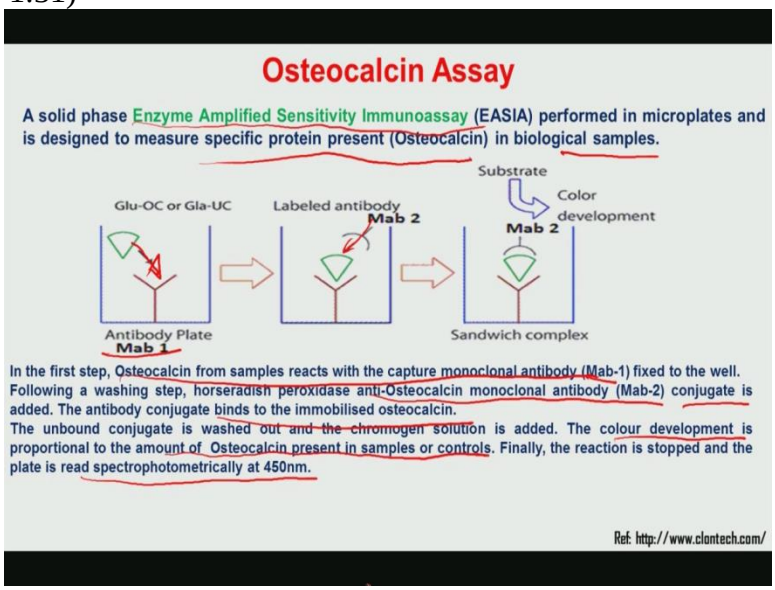

So. So here this is the one one particular assay is performed that is amino assay. That is enzyme amplified sensitivity immunoassay that is called umm. This particular amino assay is performed in microplates and designed to measure specific protein present that is osteocalcin biological samples. So like before what you do is that you first group the cells on a substrate or a culture plate and then osteocalcin from samples reacts with that capture monoclonal antibody that is Mab-1 as shown here.

So essentially it gets tagged here and then comes this Mab-2 that is this anti-osteocalcin monoclonal antibody two and their conjugate is added. This antibody conjugate then binds to the immobilised osteocalcin. So first is that you have to immobilise osteocalcin and then you have to add this Mab-2 and then unbound conjugate is washed out during this essay. And then a solution, a specific solution is added and the colour development is proportional to the amount of osteocalcin present in the samples on the controls.

And then it is essentially determined spectrophotometrically at 450nm. So this whatever assays I have discussed so far, these are typical colorimetric assays. And each assay certain optical density changes is measured at a specific wavelength using a spectrophotometer. And this any. And then change in the optical density or colour development is attributed to either specific protein or protein in the samples or it is (())(3:22) or or it is a signature of some viable cells or dead cells in the solution.

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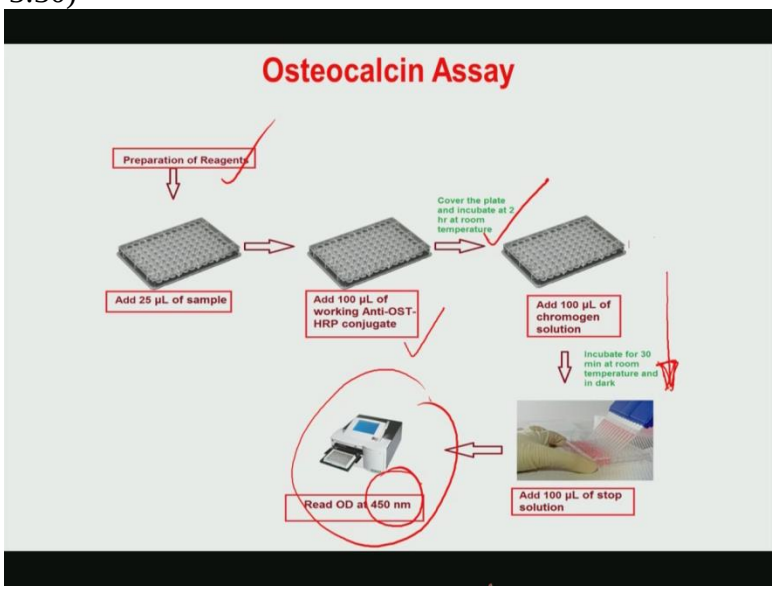

So this this is except some specific details that more or less this methodology of this assay is similar to some of this assays I have discussed before. You have to first prepare the reagents then add some specific amount of the sample like 25 micro litres and so on. Then you add this Anti-OST-HRP conjugate then cover the plate and incubate for two hours in this room temperature. Most of this procedures are essentially very standard procedure.

So there is no scope of any modification in this well established protocol or procedures that I am discussing in the context of all these assays. And finally this osteocalcin expression is read at is determined at 450 nano meter of wavelength.

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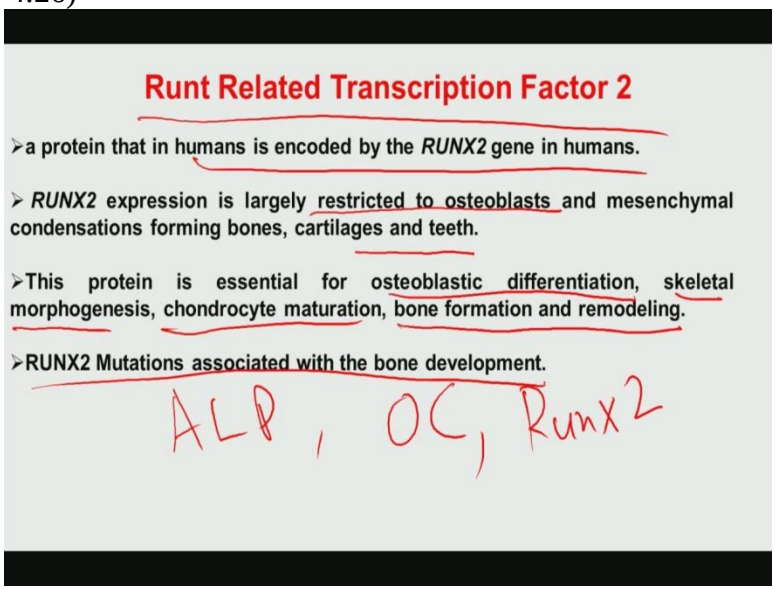

The fourth one so far I have done ALP. Then I have done osteocalcin and this is the third one that is Runt related transcription factor 2 or in brief it is mentioned as RUNX2. So this is a protein in humans, it is encoded by the RUNX2 to gene in humans. And this RUNX2 expression is largely restricted to osteoblasts. So essentially as you see that all these expression is mostly related to specific cell size bones like osteoblast and mesenchymal condensation, bones, cartilage and teeth.

And this protein is essential for osteoblastic differentiation or skeletal morphogenesis or chondrocyte maturation. So if in the context of this human mesenchymal stem cells if you see that when they will differentiate into three different lineages like osteogenic lineage, like chondrogenic lineage or miogenice lineages so along this lineage when it degrades differentiated differentiated so there this RUNX2 expression can be monitored to confirm the how this differentiation process is progressing.

As well as this RUNX2 protein is essential for the bone formation and remodelling. And RUNX2 mutations are associated with this bone development also.

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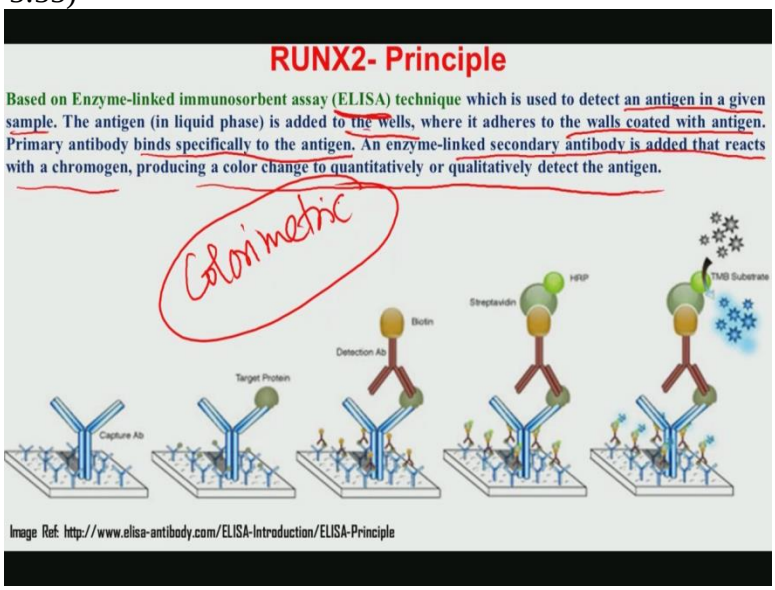

So what is the principle of this RUNX2? This is again based on a different assay. This is called enzyme-linked enzyme-linked immunosorbent assay like ELISA. So this is the abbreviated form of ELISA which biologists, they use very regularly. It used to detect an antigen in a given sample and this antigen in liquid phase is added to this culture well plate and it adheres to the walls coated with its antigen. And so it is a primary antibody that specifically binds with this antigen.

And this enzyme-linked secondary antibody is added then reacts to reacts with a chromogen producing a colour change to quantitatively and qualitatively detect the antigen. Essentially again it is a colorimetric assay and so it ends up with this colour change and this colour change you can determine quantitatively.

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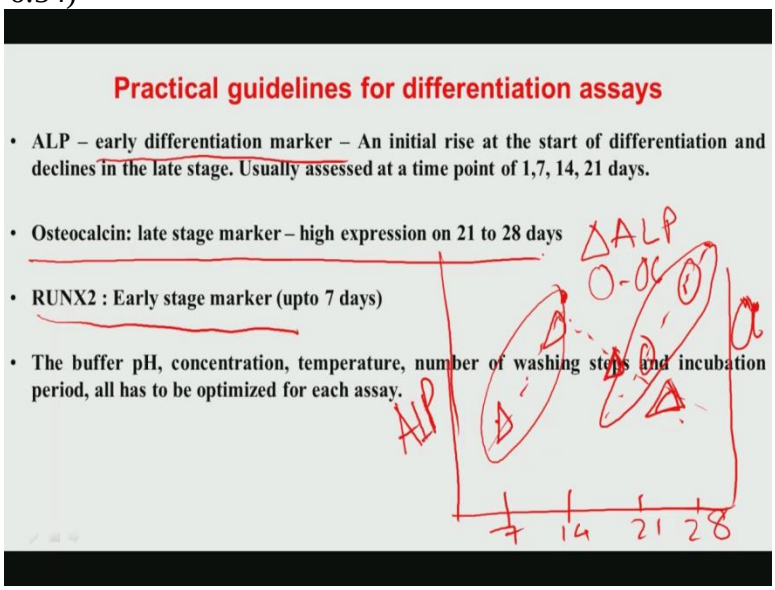

So this this three assays that I have done for this or I have discussed this module as well as previous module. So let me summarise that. So ALP as I said is early osteo differentiation marker. RUNX2 also is early differentiation marker. So mostly if you plot this expression as a function of culture time point in culture , Let us say this is the ALP and this is the osteocalcin. So suppose this is day 7, this is day 14, this is day 21, this is day 28. One would expect that ALP expression increases in the initial stage and thereafter as you go to the third day and fourth day, this ALP expression may go down.

And when it is going down, then one would expect that osteocalcin expression is go up. So this circle essentially means osteocalcin. This triangle essentially means ALP. So in a nutshell what I'm trying to explain to you that if one measures this ALP and osteocalcin expression are different in time points and culture one would essentially see that if there is osteoblastic differentiation is taking place then this ALP expression would increase during the initial stage like after 2 weeks in culture.

And if if I am measuring this ALP expression because of your lack of knowledge in cell biology then you would see that this ALP expression will decrease in the third and fourth week. And during this late stage it is osteocalcin expression which will pick up and this osteoblastic and osteocalcin expression will increase in the late stage of differentiation. So that will clearly give you a signature that yes indeed that osteoblastic differentiation is taking place because the two

signatures of this early ALP expression early ALP expression increase and as well as the late osteocalcin expression increase.

So that is why I have mentioned it very clearly that late stage marker that is high expression on 21 to 21 days. The buffer pH, concentration, washing steps etc incubation period, all has to be optimized for each assay.

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Okay. Now next set of assays that I will briefly mention. May not be in in that much details. that I have done for this earlier 5-6 assays. And this is called Immunofluorescence assay. I will just cover the basic principle of this thing first.

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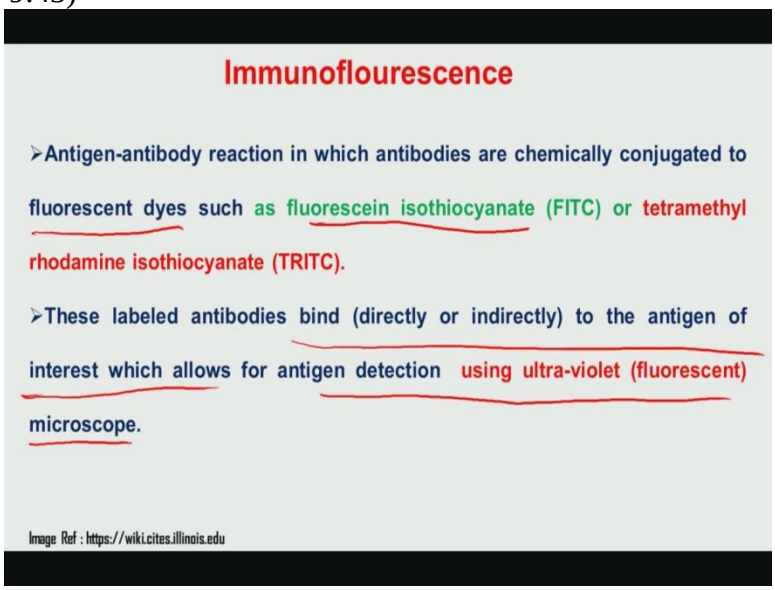

So it is an antigen-antibody reaction essentially wherein these antibodies are chemically conjugated to fluorescent dyes. So this fluorescent dyes can be fluorescin isothiocyanate like FITC stop so FITC conjugated FITC will be tagged to certain antibodies and this labeled antibodies bind to the antigen of interest and which allows antigen detection using either ultra-violet fluorescent microscope. So you can use the same fluorescent microscope in this immuno fluorescence while using the immuno flourescence assays.

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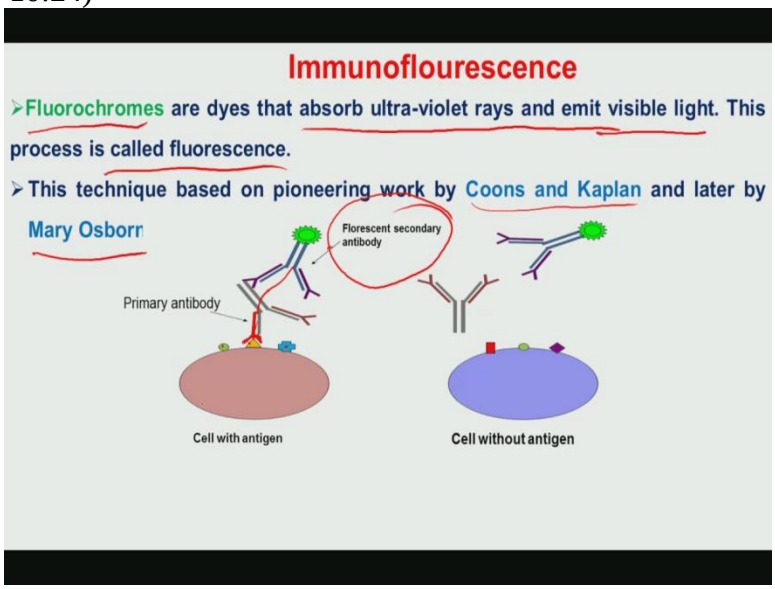

Little bit more details like here Fluorochromes as I mentioned before that fluorochromes are essentially dyes; specific dyes which absorb ultra-violet rays and emit visible light and this process is called fluorescence. There is a clear (())(10:43) difference between these two. And this technique is based on this some of the scientists pioneering work which include Coons and Kaplan and later by Mary Osborn.

So this is that your cell membrane and then you have a primary antibody shown here. And this is the fluorescent secondary antibody. Now what, when you add this things that cells with specific antibody will now be tagged with this primary antibody. So this opportunity will be absent if the cell does not have (())(11:14) of that particular antigen.

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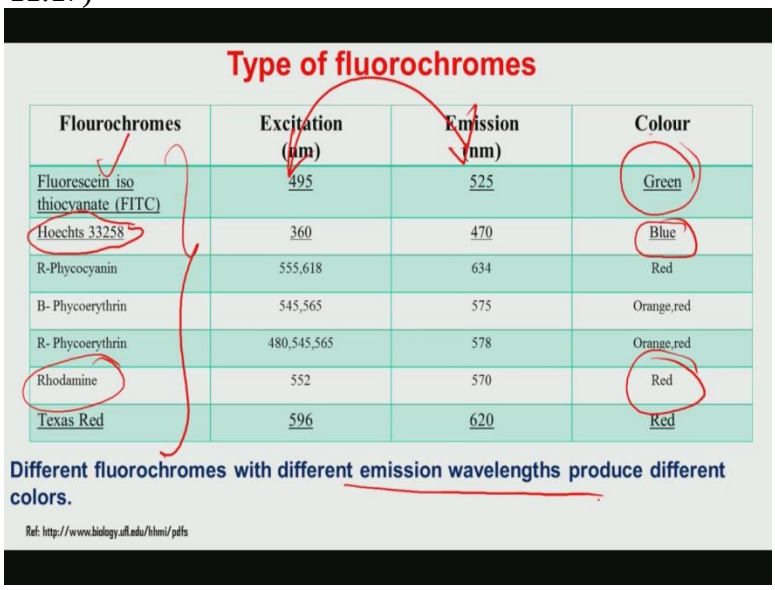

What is the typical excitation and emission wavelength as you see in each case; in case of this using the flourochromes which I mentioned before that emission wavelength is always greater than excitation wavelength. So that flourochromes will be excited at a shorter wavelength than that which it will eventually emit.

So these two things that is again you can see very clearly here. So these are the list of different flourochromes and this flourochromes they are excited at different different excitation wavelength and it will emit different wavelength; larger wavelength. And it gives a different

colour spectrum like for example this particular flourochrome which is Hoechts 33258 then it will give blue in colour.

Texas red or Rhodamine on the other hand will give red in colour. FITC that Fluorescein iso thiocyanate that will give green in colour. So this is a different flourochromes with different emission wavelengths will produce different colours. So it is not possible for a student to remember the excitation and emission wavelength for a so many number of different kind of flourchromes but certainly those with circles one can try to remember.

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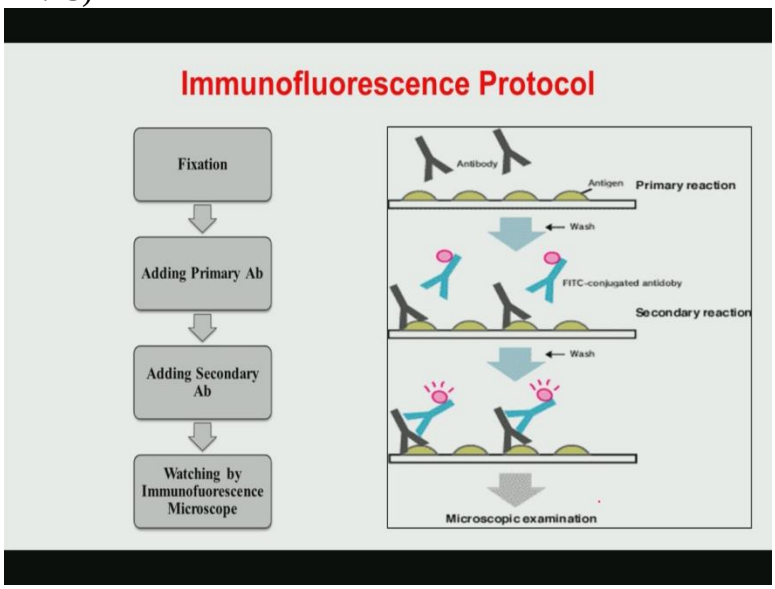

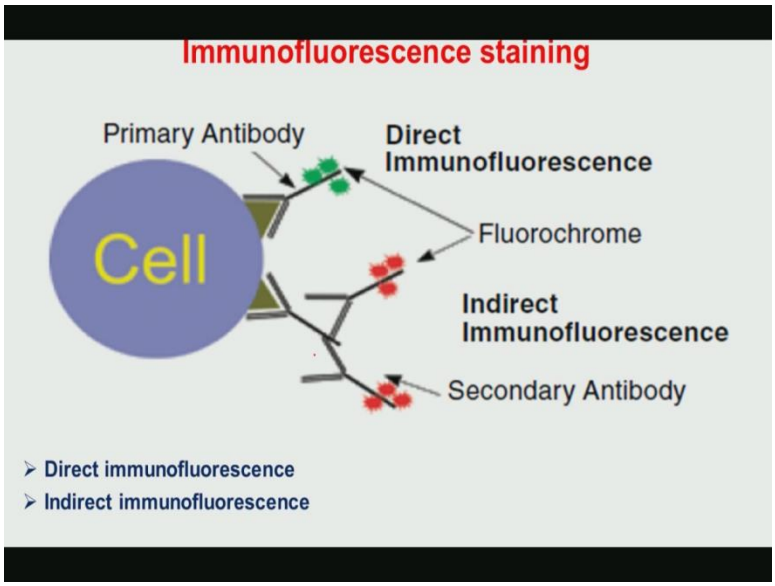

This is little bit more details of this protocol which I would like to skip umm. Then we will go to immunofluorescence staining. So as I shown you before that you have a primary antibody which is added and in case of direct immunofluorescence then you add this fluorochrome which will be tagged here. And in case of indirect immunofluorescence you add a secondary antibody and then again the same tagging phenomena will take place using the secondary antibody also.

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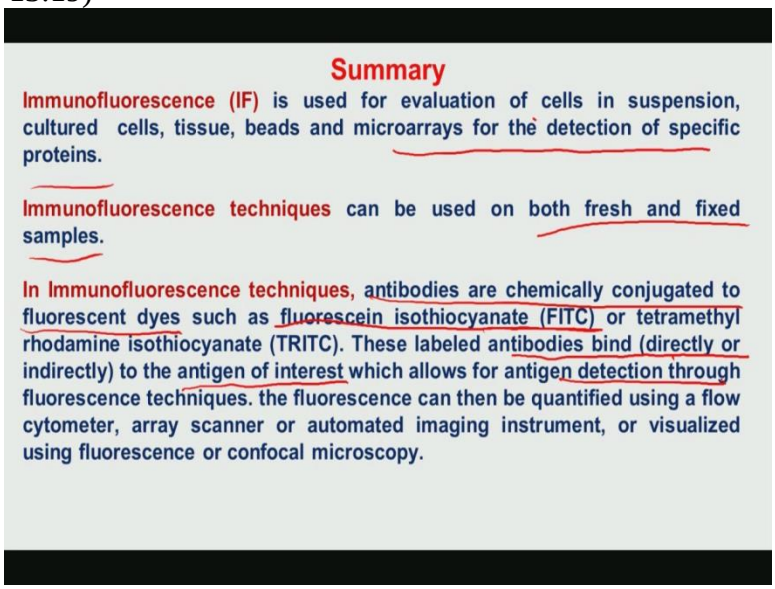

So in the summary this immunofluorescence is used for the evaluation of cells in suspension, cultured or microarrays for the detection of specific proteins and immunofluorescence technique can be used for both fresh and fixed samples. And in immunofluorescence techniques that antibodies are chemically conjugated to fluorescent dyes.

And this fluorescent dyes are shown a few fluorescent dyes are shown and in the form of a table where that excitation and emission wavelength and then colour that they are expected to they are expected to develop those things are mentioned.And other things it is mentioned here in this summary slide that after this antibodies are appropriate level they bind to the antigen of interest which allows for antigen detection through fluorescence techniques.

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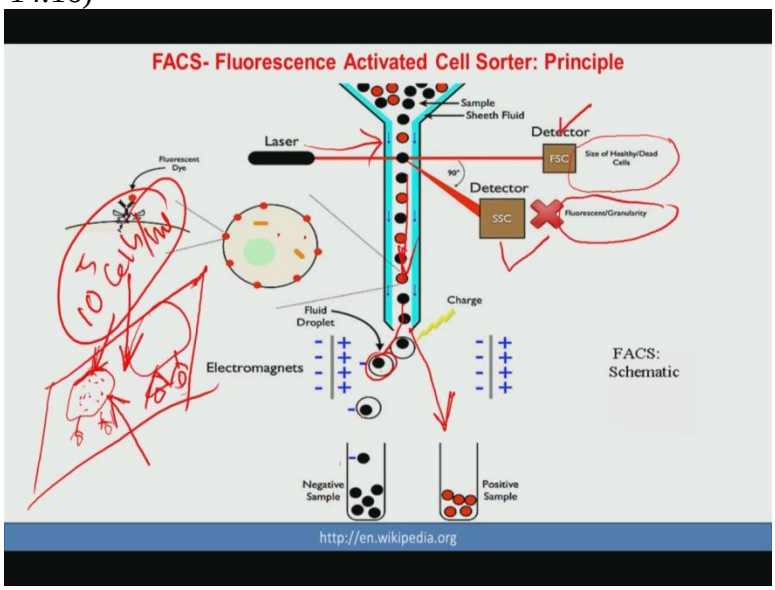

Okay. So one of the other techniques which is kind of more widely used in this biomaterials or which is now currently being used in biomaterials research for more quantification of the different cell fate processes. Now the cell fate processes I have mentioned before like cell division, cell migration, cell proliferation and all those things. So the for this quantification this facts analysis is used quite a lot. Just to give you an idea like when you do some two dimensional cell culture on a sample.

So you have this cells will spread here and the cells will now there is a 10 to the power 5 number of cells which will come and then they will tend to adhere on the material substrate. So this is a large number of cells 10 to the power 5 number of cells per mili litre and then when you add this so large number of cells to a biomaterial substrate then you will get. You will use certain assays like MTT or LDH or WST1 all these kind of assays they will produce the results in the bulk in a sense that they will tell you that this much fraction of the cells are live or this much fraction of the cells are dead.

But you do not have any access of individual cell specific information for example it will not give you that exact number of the cells that exactly this many number of cell in reality are live or this much number of cells in reality are non living. Now those kind of precise quantification is possible using the fluorescence activated cell sorter where a cell suspension is made to flow

through a tube and these in this fluorescence cells cell sorter tube and then a laser will focus on this particular cell suspension as it goes through the tube.

Then there are two detectors. One is that FSC forward scatter forward scatter then will be side scatter. And both these detectors are kept at 90 degree orientation and then forward scatter will tell you that size of the healthy or dead cells whereas side scatter will tell you will give you that fluorescence or granularity. Now once it goes through this column then it then it will and use certain fluorescent dyes to start with and then because of the fluorescent dyes you use then it will be the cells will be attracted either towards this positive electrode or towards the negative electrode.

As a result few cells will be collected in that positive side and other cells will be other cells will be collected at the negative electrode side.

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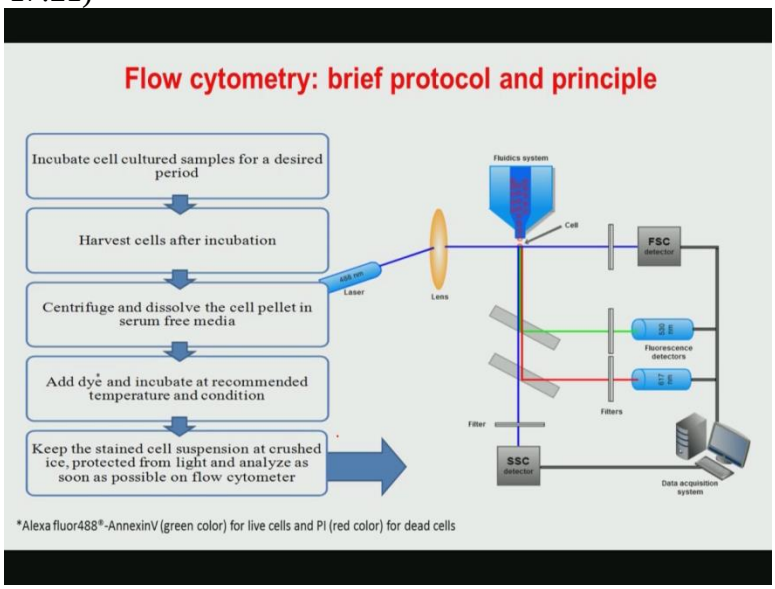

So then this particular this particular technique is used to quantify that what is the fraction of cells are at different stages in the cell cycle. Umm. Or what whether what is the fraction of the cells are live and what is the fraction of the cells in apoptosis or necrosis stage.

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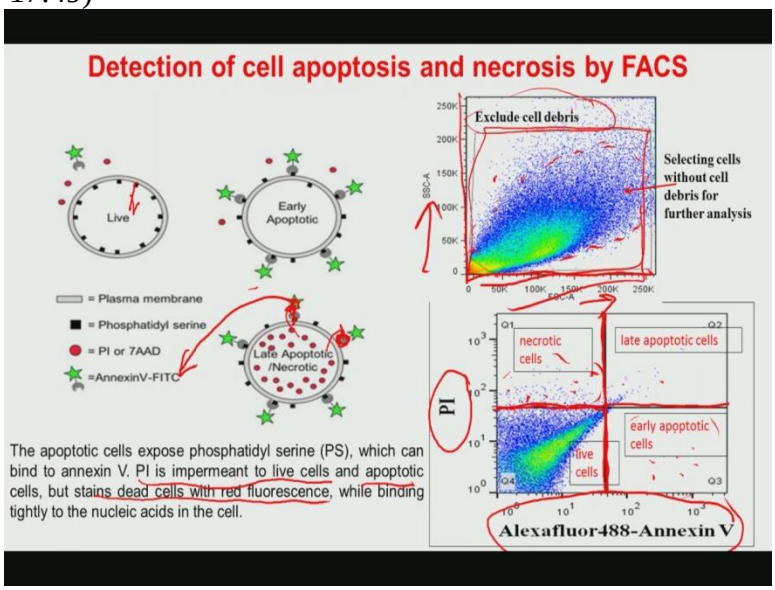

Now for that first we will discuss that use of this fluorescent flow cyctometer to detect the cell apoptosis and necrosis. So typically that FACS will give you a plot like this so as it is shown here. Along the x axis is forward scattered, along y axis is your side scattered. There will be lot of concentrated area where there is dots will be there in multitude and there is some area where this dots are very discontinuous or where this is not a very dense dots.

First of all each dot in any FACS plot contains the information from one single cell. So essentially if you now quantify or if you now do a proper getting like the way it is shown here. So you exclude the cell debris then you then you get this data. Then after this gating if you do proper analysis to see. What is the fraction of the cells lie in different quadrants.

So inter FACS plot is now divided into four four quadrants depending on whether it is low PI expression or low Annexin expression or high Annexin expression. Now to start with in that live cells phosphatidyl serine is typically present towards cytoplasmic side that is that is towards the cytoplasmic side. Now when cell is in necrosis stage or late apoptosis stage then there is a flip flop mechanism which helps the phosphatidyl serine molecule to translocate to the outside of the cell membrane.

Now if you add a specific dye then what will happen which can bind to this phosphatidyl serine which is shown here annexin FITC then annexin FITC can conjugate with this phosphatidyl

serine. So therefore higher the tagging, more will be the annexin expression. Okay? So therefore if that more if if certain region shows large number of dots with high annexin expression, that essentially tells you that this cells this fraction of the cells have phosphatidyl serine molecule which are located now outside the plasma membrane towards the extra cellular space.

As a result those cells are not live cells anymore, they will perhaps go to this more earlier apoptosis to the late apoptosis stage. So this is the basis for detecting this what is the total fraction of the live cells or what is the total fraction of this early apoptosis or necrosis cells. So propidium iodide and this similarly is impermeant to live cells and apoptotic cells but stains dead cells with red fluorescence. So therefore higher propidium iodide expression will also tell you that this number of cells are dead cells.

So therefore all the dots which shows that large propidium iodide expression essentially not alive and then they are essentially necrotic cells. So each time you use this kind of fluorescent FACS analysis, this plots are to be meaningfully analysed so that you can extract that that quantified results to essentially tell that what is the fraction of the cells are live or what is the fraction of the cells are in that apoptosis stage.

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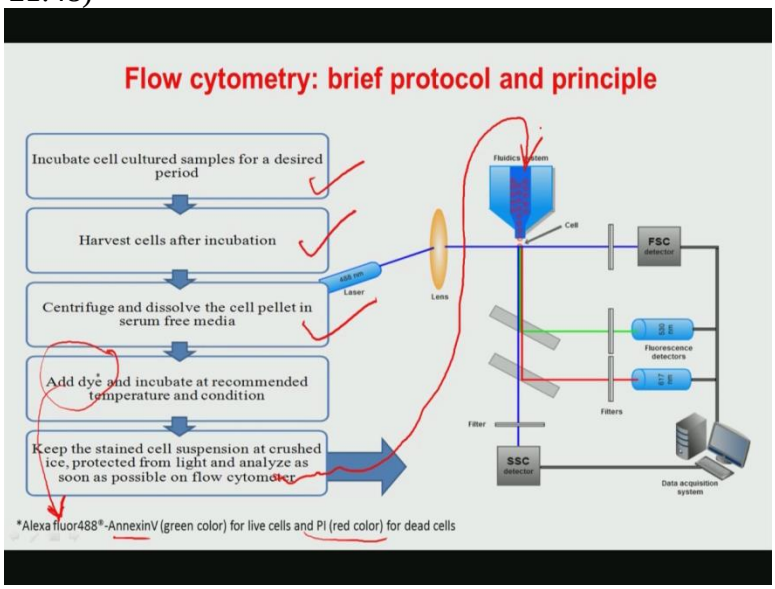

Now this this protocol is fairly simple. Like you incubate the incubate the cells on their biomaterial sample and then you harvest the cells after incubation, they centrifuge and dissolve

the cell pellet in serum free medium. Then add different dyes. Now this dyes would be specific for your study.

For example if you want to quantify the apoptosis necrosis or cell viability then you have to use that Alexa Fluor488 that is that Annexin green colour. Add propidium iodide for these dead cells. Then after you add this dead and again the dye again you have to incubate for the recommended conditions and then keep the suspended cell suspension in crushed ice and then directly add to the fluorescent Flow cytometer.

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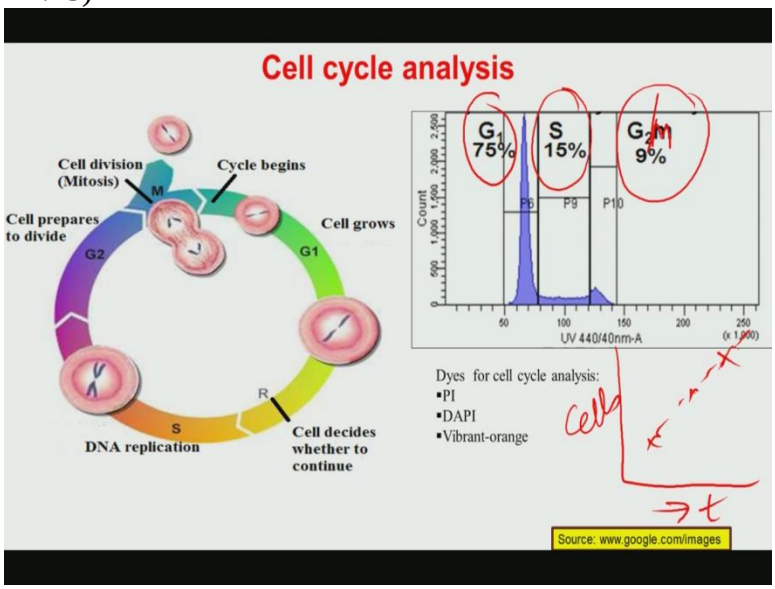

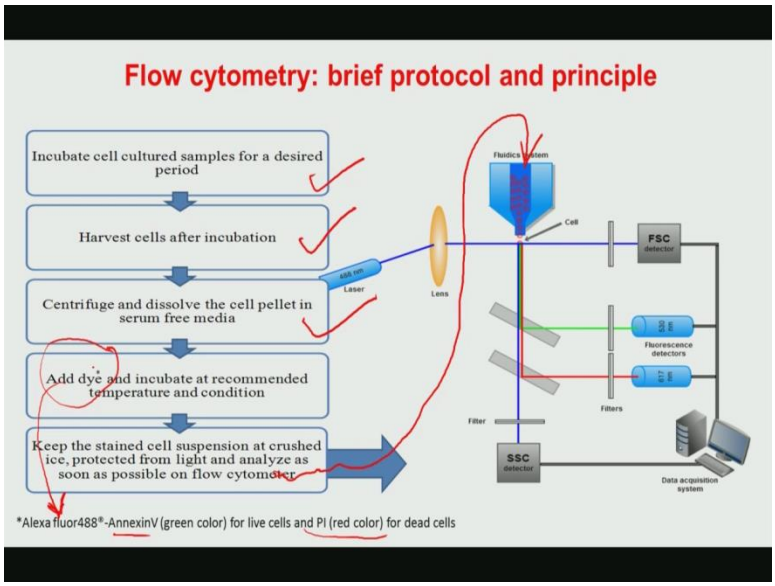

Ok. Now the other things that we can we can see that how how useful this Flow cytometer is in the context of cell cycle analysis. Now when you do typical entity assay and all, you can say that ok whether this number of cells are increasing at different time points in culture so it will certainly indicate that when this number of cell, number of number of viable cells it increases in the time of this is the cells permit area whatever. That whether this number of cells increases as you grow the cells for longer and longer culture duration that certainly shows that cells are growing that means cells are dividing. But you do not have any clue from the simple entity assay that what is the fraction of the cells are there in different stages of their cell growth.

You just have a rough idea overall idea that simply this number of cells are growing on while being attached to the biomaterial substrate. So if you know now you know from my earlier modules that cell cycle goes through certain checkpoints if you remember correctly and then it goes from G1 to S to G2 and then finally M. So each time at the checkpoint there are certain conditions had to be satisfied then only the cell will go in an irreversible manner from G1 to S and subsequent S to G2.

And if I the cell is stuck in certain checkpoint they are not able to go from to the next stage then I have mentioned to you that the cell would essentially initiate it is own suicidal mechanism and would undergo apoptosis what is nothing but programmed cell death. Now having having said all those fundamentals now we would like to know in a given cell population, what is the fraction of the cells in the G1 stage. What is the fraction of the cells in S phase and what is the cell fraction of the cells at the G2 and M phase.

And this quantification is possible if you use that FACS analysis and again as I said before that for each of the cell fate analysis you have to use different dyes .Now what all different dyes are to be used for that you can always use certain textbooks and then which may not be very easy to remember for different cell fate processes but the protocol remains almost similar. Like the way I have mentioned here that after standard or conventional culture you have to add some dye. Then you have to incubate and then you have to store it and then immediately put it.

Now whether you want to use this typical cell viability or necrosis and apoptosis you have to use different dyes. If you want to study the cell growth processes or cell division or cell cycle process cell cycle then you have to use different dyes so essentially for each time you have to use different dyes and this dyes once you use that dyes then there are certain scientific rational which will tell you that progressive reduction of the specific dyes will give signature of certain processes.

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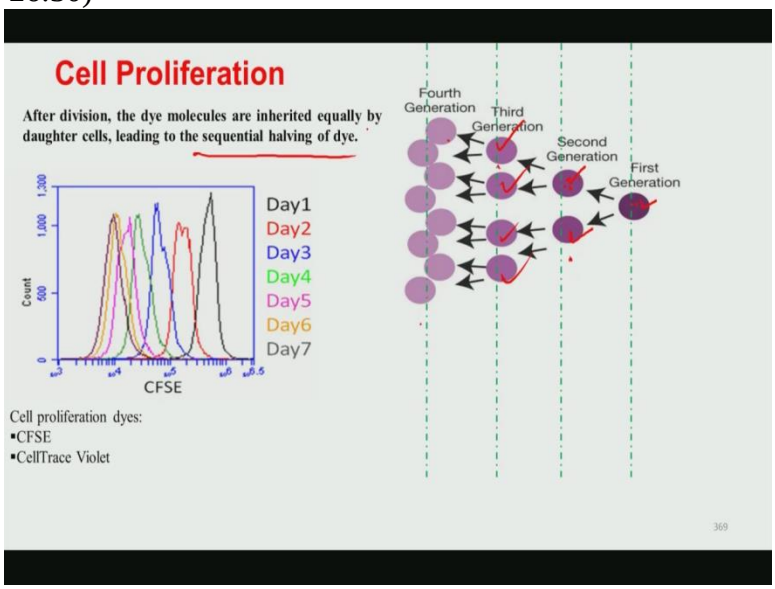

Now in the context of the cell proliferation you see that first generation 1 to 2, then third generation 2 to 4. Then fourth generation 4 to 60. So like that one to two so each time if you use certain dyes which will gets their intensity gets halved like because that one cell goes from 1 to 2 that means now this dye is absorbed by two cells instead of one cell.

So intensity if you measure, it should be gets halved. Now whatever intensity you have measured here 2 then when it goes to 4 then it will be subsequently halved again. So it will be like one by two, one by two, one by two like that the intensity will be reduced and essentially the sequential having of dye will tell you that when cells are proliferating one at different time points in the culture. So this is certain rational you have to know a little bit of biology and you have to see that how to read this kind of data to actually interpret what kind of cell fate processes the cell is undergoing.

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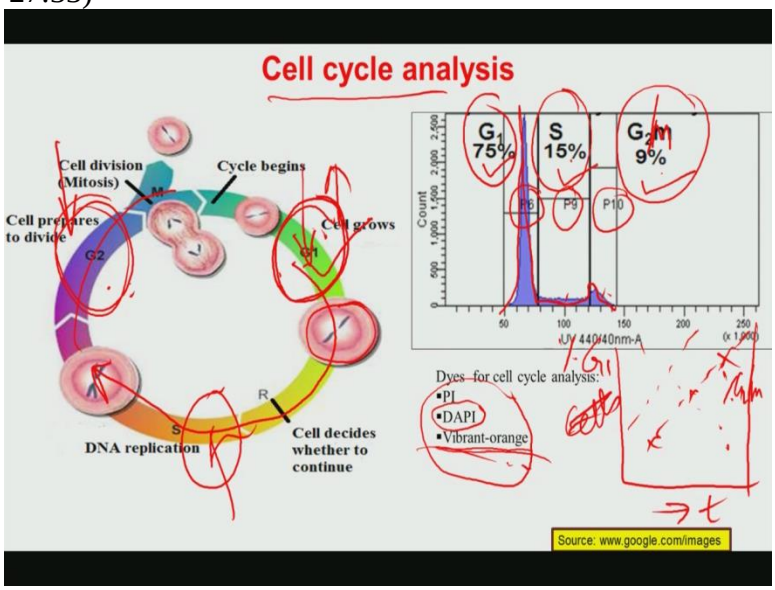

Now coming back to the cell cycle analysis now you get these kind of plots now this and these plots are essentially coming from different getting like P6 getting P7, P9 getting and P10 getting. Now if you do this particular analysis this first peak will always give you that fraction of the cells which are in G1 . Then if you see the second one that will give 2S and then the third one is G2 and M. So here for example it is quantified as 75% as G1 phase, 15% in the S phase and 9% is the G2 and M phase. Almost it is if you add it up, it almost coming out to be 99% close to 100%.

Now what are the other thing that I mentioned? I want to mention that during different time points in culture if you see that your if now if you plot one side is number of cells in percentage G1 and another side in the percentage G2 or M. Now if you see that G1, the fraction of the G1 cells is increasing and fraction of the G2M phase is decreasing then you can see very well that cells are stuck somehow in the check point because so that this fraction of the cells which are present in the G1 phase increases and fraction of the cells in G2M phase decreases.

That means cells are not able to cross this checkpoint come to the G2M phase. So if in one stage the cell numbers increases, in another stage cell number decreases. That clearly shows you signatures of that the cells are not able to divide properly over different time point in culture and somehow cells are getting stuck at the intermediate stage either in S phase or in G1 phase and they are not able to go to the G2 or M phase.

Now there are different dyes which are used for the cell cycle analysis which are used which are mentioned here PI propidium iodide. I have mentioned in the last to last slide that PI is impermeant to this live cells. Now DAPI I have mentioned you in some of the earlier modules. The DAPI is this fluorescent which directly interconnects to the DNA of the cells live cells.And then the third one is the vibrant orange. So all three dyes are used for different purposes to quantify the cell cycle analysis.

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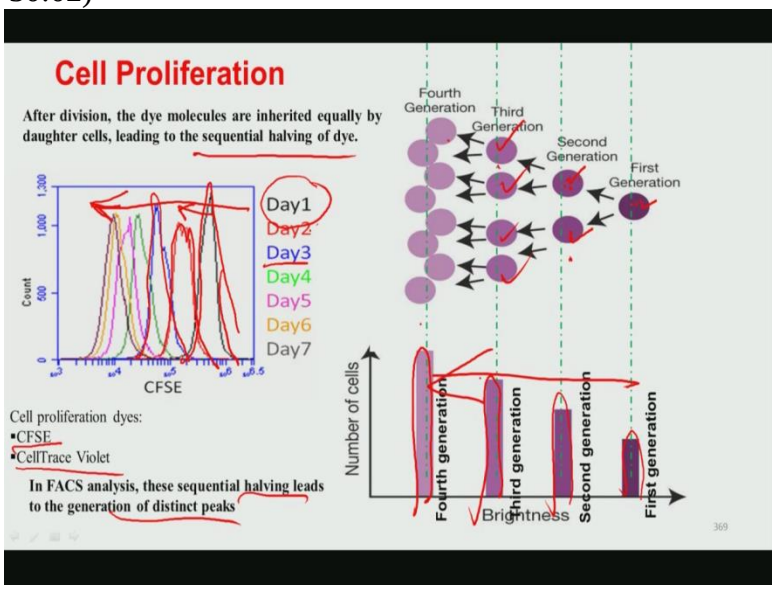

Now cell proliferation, the dye which is mostly used at CFSE and Cell Trace Violet and then as I mentioned to you that if you see this black one is the Day 1. That is that CFSE intensity now in the Day 2 it is shifting towards the left side as you have seen progress this is the Day 2. In the Day 3 that is the blue one this is shifted again further left.

So all the time if you use the CFSE dyes, their intensity is going towards the going towards the left and in in a in a way that is there sequentially sequentially having of the dye is taking place that shows that this is the and these are the indications that the cell proliferation cell proliferation is taking place. The sequential of the having essentially leads to the generation of distinct peaks. So you have a first generation. You have a second generation. You have a third generation and you have a fourth generation and you can see that their peaks are going towards more and more left.

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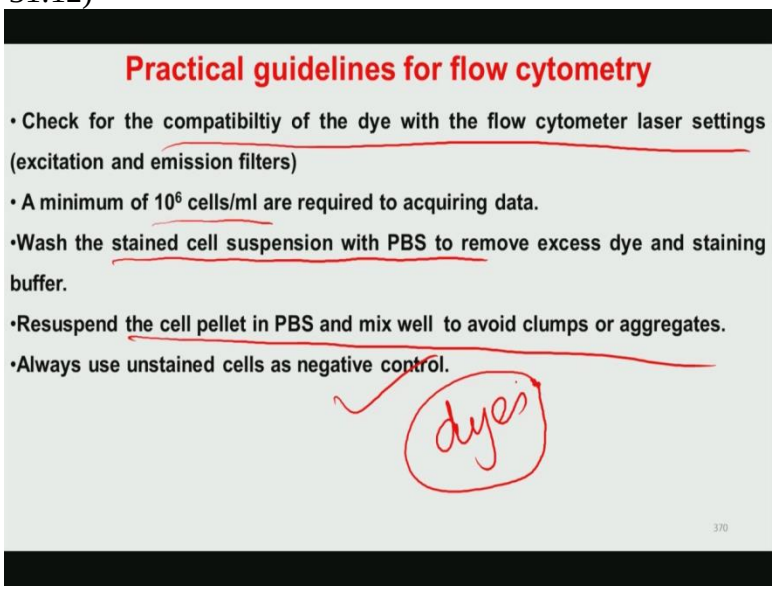

Okay. So certain guideline for the flow cytometry analysis. First you have to see the compatibility of the dye of flow cytometer laser settings like excitation and emission filters and typically 10 to the power 6 cells per milli litre are required for to acquire data and one has to wash the stained cell suspension with phosphate buffer saline to remove excess dye and staining buffer. And you have to resuspend the cell pellet in the PBS and mix well to avoid clumps or aggregate.

And it is recommended to use always unstained cells as the negative control in that FACS analysis. And then other thing that I have mentioned in the dyes, these dyes are different for the different aspect of the cell fate processes that you would like to study.

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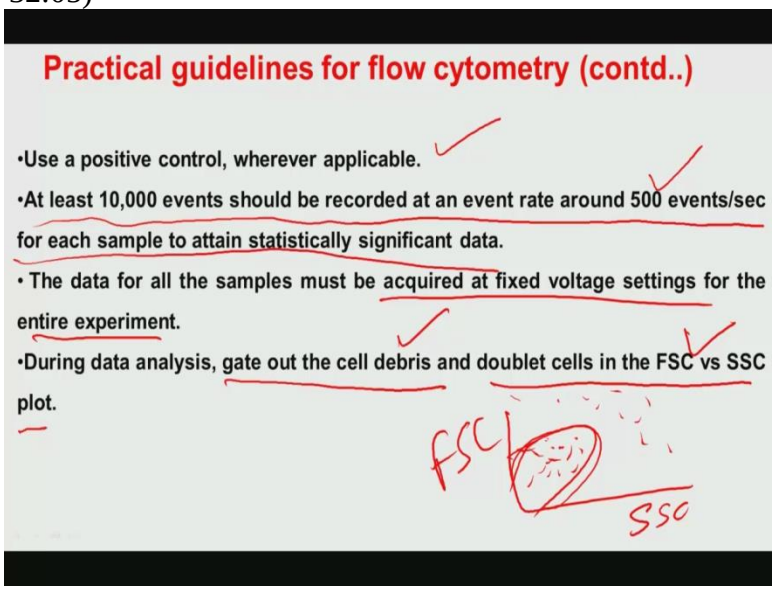

Umm. Other practical guidelines of this flow cytometry that use always a positive control wherever applicable. And the advantages or the clear advantages of the flow cytometre over any other existing techniques is that at least ten thousand events. Ten thousand means each dot point you remember in the flow cytometre plots at least 10,000 events can be recorded at an event rate of 500 events per second. So now you can understand that how fast the flow cytometre as a technique to detect all the cell fate processes.

First of all in all this conventional biochemical assays, you cannot detect or you cannot record cell specific manner or individual cell specific manner information which you can directly get in the flow cytometry. Secondly you can get a huge number of events like ten thousand events which can be recorded with per second you can essentially record 500 events and data for all the samples must be acquired at a fixed voltage setting for the entire experiment.

And during the data analysis I have mentioned in one of the slides you have to first get out the the cell debris or you have to get actually meaningful meaningful data points which shows very concentrated at very dense data points in the forward scattering versus side scattering plots.

If you remember this is your FSC this is your SSC or vice versa you have this lot of this dotted points and some of the data dotted are very discontinuous so wherever there is a concentration of

large number of dot points so those things we have to get it first. So I think I will stop now and we will continue in the next.