Experimental Biotechnology
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Lecture – 37
Flow Cytometry

Hello everybody, this is Dr. Vishal Trivedi from department of bioscience and bioengineering IIT Guwahati and within the cell biology so, far what we have discussed we are discussed about the cell culture technique, we discuss about the differentiation of the different types of cells. And then within the cell we also discuss about how you can be able to isolate the different organelles, utilizing the differential centrifugation or the instigation centrifugation.

Apart from that, we have also discussed about in depth different microscopy techniques, whether it is the phase contrast microscopy, or the fluorescent microscopy or the electron microscopy. And in detail we have discussed about the different types of protocol as well as the procedure what you have to follow to solve to process a sample for the fluorescent microscopy.

Phase contrast microscopy, electron microscopy whether it is the scanning electron microscopy or the transmission electron microscopy; and apart from that, we also discuss some of the crucial experiments. So, now, today we are going to discuss about different topic and the topic is the flow cytometry. So, what is the use of the flow cytometry, you can imagine a situation where you are actually trying to handle a complicated tissue.

For example, if you are handling with the tissue and you want to analyze all the cells, then you are actually looking for a system where which can be able to analyze them separately, and you do not want that them to be separated simply by the denstigated centrifugation or the differential centrifugation. So, let us discuss that and we will start with the composition of the different tissues.

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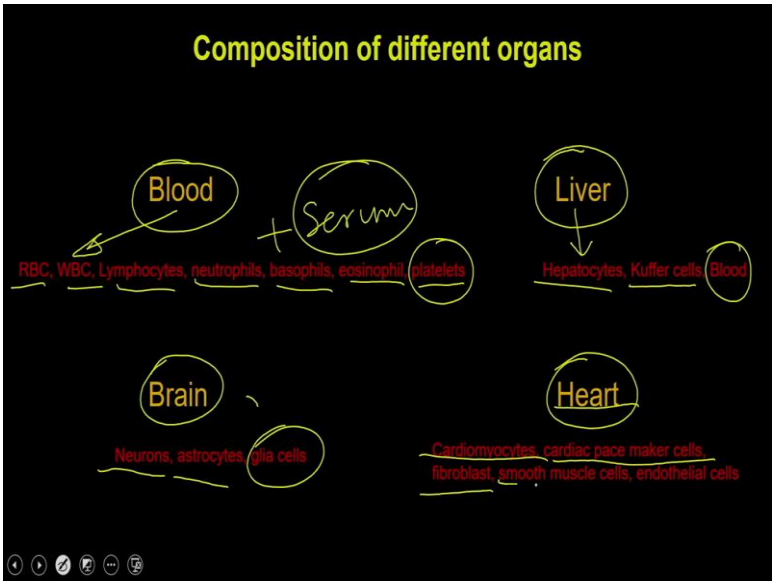

So, as you can see, I have taken an example of the some of the tissues what is really in a human body, whether it is the blood, liver, brain or the heart. And what you can see is that the for example, the blood like the blood is made up off of RBC, WBC. Within the WBC you have the lymphocytes, neutrophils, basophils, eosinophil and platelets. Apart from that, you also have the serum component.

So that is the A granular component or the A cellular component. So that is we are going to be keeping it separate. And like for example, in the liver, you have the hepatocytes, you have the kuffer cells and then liver is also having the extensive supply of the blood. So you also can go in to have the blood cells. Similarly in the brain, you can have the neurons astrocytes, as well as the glia cells.

Apart from that, you also might have the some of the fibroblasts and all other kinds of microbes, macrophages and all other kinds of cells. And then talking about the heart, you can have the cardiomyocytes you can have the cardiac pace maker cells, you will have the fibroblasts, you can have the smooth muscle cells, and you can have de endothelial cells, which actually are responsible for making the arteries as well as the wills.

So, as you can see, all these tissues are very complex composed of the different types of cells. And you can imagine a situation where you are interested to study the self, but you do not want to disturb them, or you do not want to hurt these cells with by going through with the extensive cell certifications or some of the purification techniques. So, how you can be able

to achieve that you are looking for a system which actually can separate the cells under the flow and then you can be able to individually study these cells.

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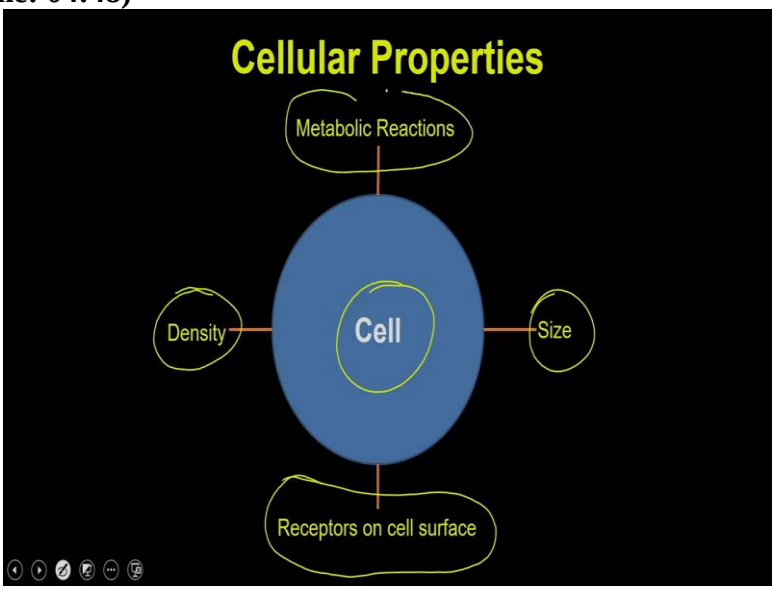

So, when you are want to study individual cells, or when you want to distinguish a particular cell from the other cell, you have the 3 to 4 different criteria what you can use. So, as far as the cell is concerned, what you can have, you can have the shape, so, different cells will have the different types of shape, then you can also have the density of that particular cell, so, that the number of different types of organelles, what is present in a cell is actually going to composed and will responsible for the density of that particular cell.

And then you can also have the different types of receptor onto the cell surface and that also can be a criteria to distinguish one cell from the other cell. And then at the end the different types of cells may also have the differential metabolic behaviour or metabolic reactions. For example, in some cells, you might have some reactions, which is responsible for production of the free radicals.

For example, in the immune cells, you always have these kinds of things, then in some cases, you might have the calcium flux and in some cases you might have the ATP productions and all that. So, basic pathway may not maybe remain the same in different types of cells, but all the other types of cell which is responsible for giving them the functional outcomes may be different in different types of cells. So, all these 4 criteria can be exploited in a flow based essay, where you can actually be able to utilize these parameters to distinguish one cell from another cell.

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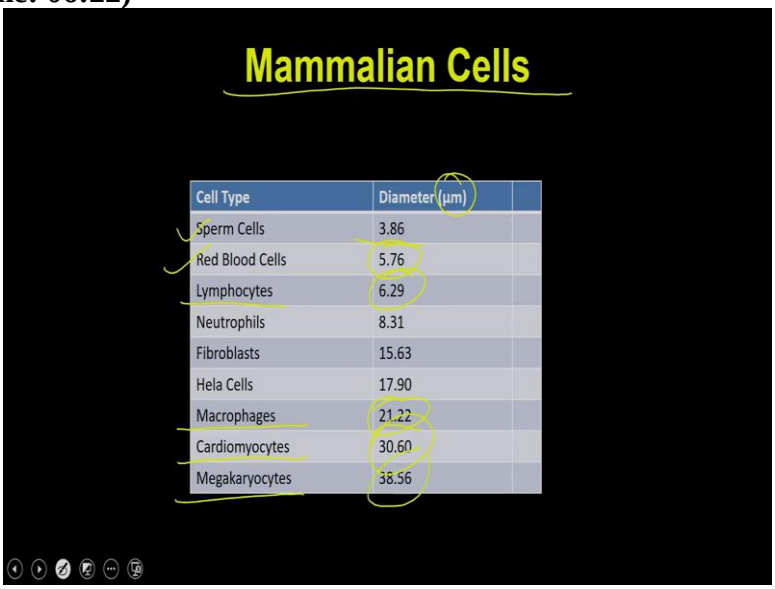

If you want to do that, for example, in mammalian cells, you have the different types of sizes like for example, sperm cells are having a size of 3.86 micrometer, red blood cells are 5.76 lymphocytes are slightly bigger 6.29 and what you can see is the macrophages are in the range of 21 micrometre cardiomyocytes are even bigger than that for you, they will be of 30, micrometre and megakaryocytes are very big.

So, they are around 40 micrometer. So, what you can see is the size in the in terms of sizes, different mammalian cells are different from each other. Similarly, the cells are different in terms of the density as well as the metabolic reactions as well as the cell surface receptor, what is present on these cells. So, these are the 4 criteria one can easily use to exploit and one can easily use to distinguish the different types of cells. So, for analyzing the cells, you are looking for a system which actually can perform these experiments these criteria for individual cell.

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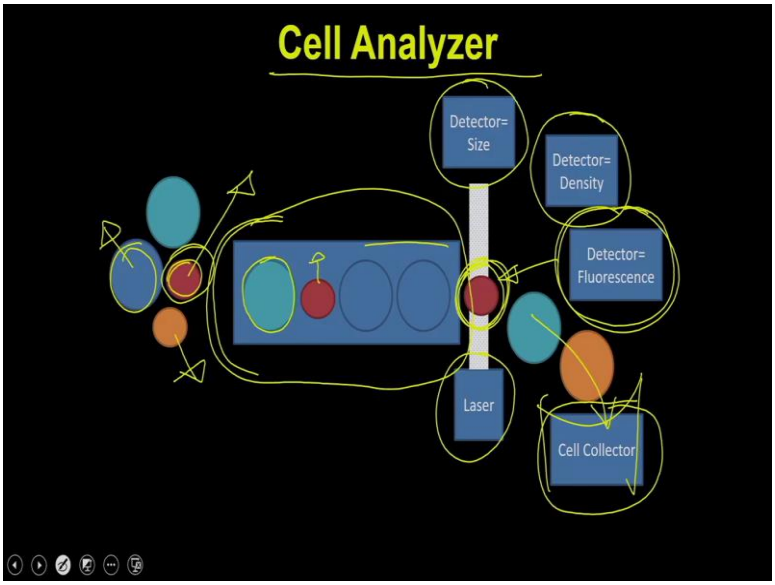

So, for that kind of thing, what you are looking for is you are looking for an instrument or you can be looking for a cell analyzer, which could be able to first you can imagine that you have a similar populations, where you have the cells of the different colors. So, what I am showing is a different color means it is going to cell have different types of metabolic reactions or different types of cells or receptors or it could be of different sizes like this is a big cell or this is a small cell.

So, irrespective of that, first you are looking for a system. So, that it actually can distinguish and analyze this cell mass and give them a single cell. And once the single cell is coming out from this tubing is could be eliminated with the help of a laser and when you illuminate this cell, you can be able to have the detectors, these detectors could detect the size of this particular cell or you can have the detector which can detect the density of this particular cell or you can have the detector which actually can detect the fluorescence.

So, from where you are going to get the fluorescence. For example, if this cell has the different types of receptors, then what you can do is you can just simply add the antibodies, which are actually going to bind to this particular cell and it is actually going to give you the fluorescence and that can be detected with these kind of the fluorescence detectors and then at the end you also require a cell collector.

So, in case suppose you want to collect this particular cell and you want to further analyze and perform some experiments, then you can actually have a funnel kind of thing and that actually can be used to detect or to collect these cells. So, this is a basic instrument or basic

infrastructure what you require it for if you would like to analyze come a complicated mixture of cells utilizing the cell analyzers. Let us see how the instrumentation looks like.

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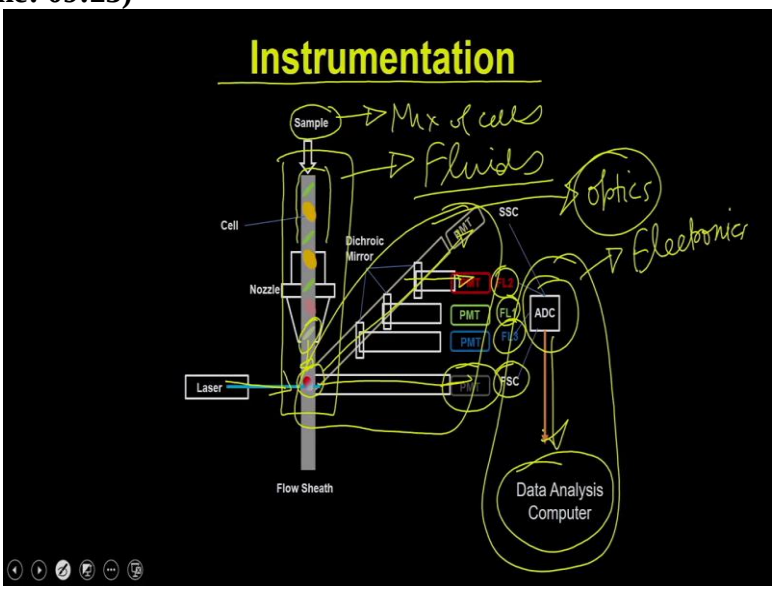

So, in a in a flow cytometer the instrumentation is very simple to that what you have is you have a sample, which is actually going to have a mixture of cells. So, once this mixture of cells are going to enter into a tube, it is going to be first focused and then you are going to have the single cell coming out from this tubing and then this single cell is going to be eliminated by a laser source.

So, once this single cell is going to be eliminated by the laser source, it is going to give the signal and these signals can be detected by the different types of detectors, whether it is the detector for the forward scanning or it is the detector for the side scanning or whether it is a detector for the different types of fluorescence channel whether it is the FL-1 FL-2 or the FL-3 are these are then going to be processed by the processing unit and then it is going to be converted into a readable signal.

And that actually is going to be analyzed by the computer and at the end you are going to see the readouts from these this particular individual cell. So, if you see this particular instrumentation what is for the cell cytometer what you have is you have the 3 components one is this particular component which is called as the fluidic components or the fluidics. So, that is the fluidics components where you have the fluids where you have the water and then this is the component which is called as is a part of the optic system.

So, that this is a system which is actually analyzing your sample or the signal what is coming out from your sample and then this is the signal system what you have required this called as the electronic system. So, that electronic system is going to convert this optical signal into a readout signal. So, it is actually going to convert that photon what is coming. What is being perceived by the optical system and then it is going to convert it into a readable signal and that is going to be shown by the computer.

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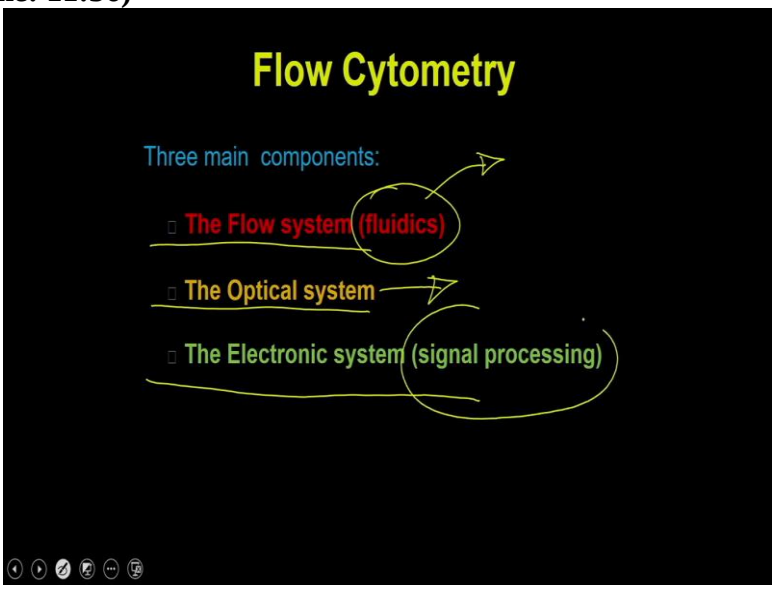

So, in a typical flow cytometer you have the 3 components you have the flow system which is called as the fluidics and the job of the fluidics is that it is going to focus the sample. So, that you can be able to have the single cell at a single at a time and then this single cell can be eliminated with the laser. So, that is a part of the optical system. So, optical system is composed of the lasers on one side and the detectors on the other side and then the third system is the electronic system or the signal processing system.

So, the electronic system is simple that it is going to convert that system signal what is coming from the optical system and then it is going to convert it into a readable signal first discuss about all these system so individually.

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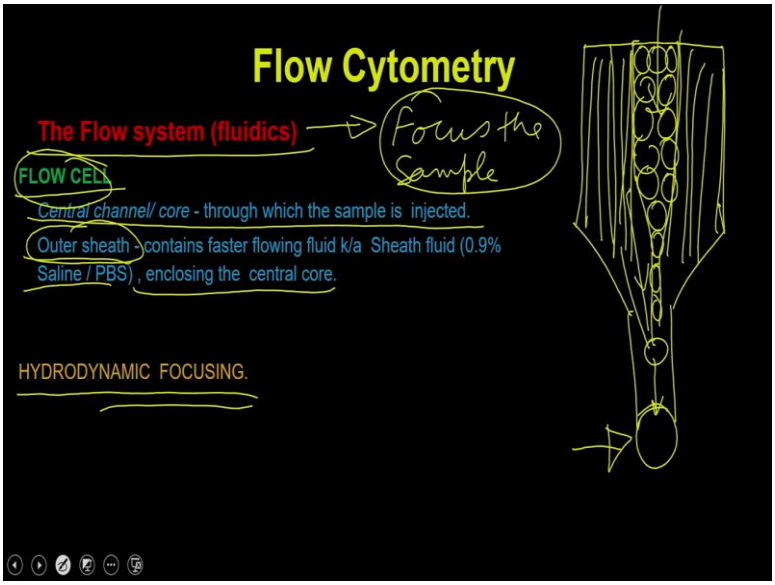

So, in the flow system, you are going to have the flow cell and the flow cell the purpose of the flow system is that it is actually going to focus the sample. So, that you are actually going to have this single cell at a time. So, this focusing of the sample is being done with the help of the sheath fluid. So, what you have is when the sample is injected into this flow cell, the flow cell is actually going to have the sheath fluid.

So, you have the outer sheath fluid and that actual outer sheath fluid actually contains the 0.9% saline or the PBS and then it is actually enclosing the central core. So, in the central core, you are going to have the sample whereas, which is actually going to be surrounded by the sheath fluid and then it is going to use the hydrodynamic process to focus the sample. So, you can imagine that you have the sheath fluid running into this.

So, this is a typical design of a flow cell. So, once the sample is entering into this flow cell, so, what will happen is it is actually going to focus this sample and then it is actually going to give you the individual cells. So, you can imagine that you have the multiple cells at the base of this particular sample, but then ultimately it is going to give you the single cell and that happens, because the sheath fluid is running on top as well as the bottom of this sample and this sheath fluid.

The speed of sheath fluid is being controlled in such a way that it is actually going to focus the sample and as a result the single cell is going to come out from this fluidic system and then this single cell is going to be eliminated by the laser. So, this is actually a typical way in

which the sample is going to be focused by the hydrodynamic focusing system and these fluids are flowing in a single channel.

But they do not mix because the speed of these fluids are very different and the density of the liquid what you use for the sheath fluid versus the density of the liquid what you use for preparing the sample is different. So that is how they are actually been flow around the sample without having any boundaries. So, if you imagine that there is a boundary, there is no boundary, the sample is also running into the liquid, this sheath fluid is also running as a liquid, but because the sheath fluid is running very fast and it is in a focused way.

So, because of that the sample is getting focused and then the single cell comes out from this particular fluidic system. So, once the single cell comes out, then these single cells are entering into the optical region, where you have the different types of source light as well as the detector lights.

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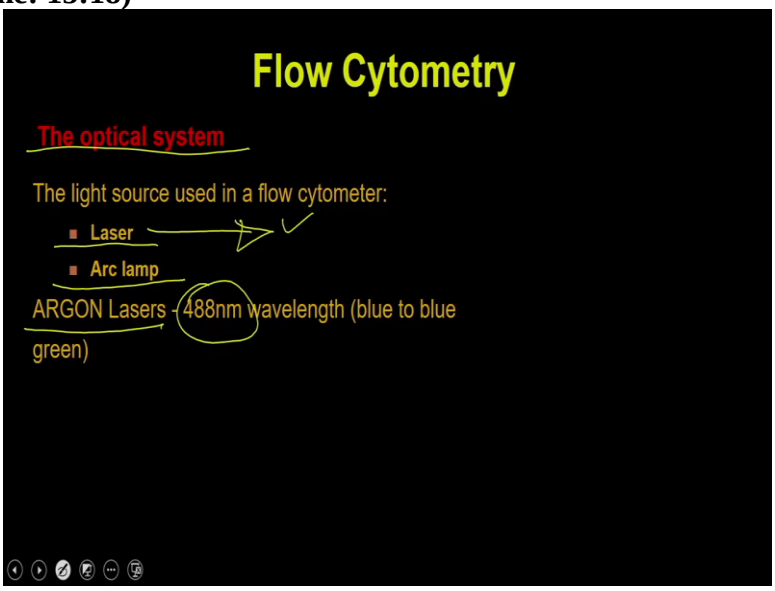

So, once they enter into the optical system, the optical system has 2 system one is the source through which you are going to use to eliminate the sample. So, you can have the laser as a system laser as a source or to the arc lamp. In some cases the people are also using the argon laser. So that argon laser is actually giving the flexibility to excite the sample at 488 nanometer laser is always been used.

Because the laser is giving you the very, very high intensity and the very high intensity and that is where the laser is preferred over the some kind of tungsten or arc bulbs because the

intensity of the bulb is always been varying whether it is you know the beginning or the end so, that fluctuation could actually be problematic for the instruments to or for the samples to you know, to get excited and some cases the lamb based excitation is also going to damage the florescence.

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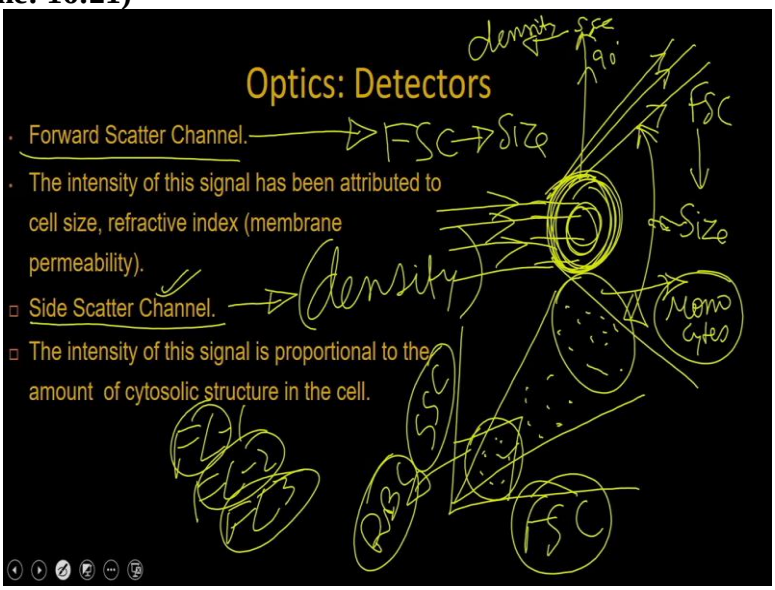

So, this is the light source and as far as the detector is concerned you have the multiple detector, the detector which is actually going to detect the forward scanning or the forward scattering or you can have the side scattering. So, what is mean by the forward scattering and side scattering is that you can imagine that you have an object and then if you are eliminating it with laser beam.

So, what will happen is once the laser beam is going to hit this sample, it is going to be scattered in 2 directions one it is going to be scattered in a cone shaped structure like in the forward directions or it is actually going to be scattered in a 90 degree angle. So, this is called as the side scattering which is called as SSE whereas, this is called as the forward scanning or the forward scattering, the forward scattering as you can see that the forward scattering is depending on to the size of this particular thing.

So, FSC is actually going to give you an idea about the size of the particle whereas, the SSC is actually going to give you the idea about the density of the molecule because as the molecule will as these laser beams are going to hit the material what is present inside the cell it is going to be scattered it by a right angle. So, if the right angle condition, if the material is less dense than the scattering.

What you see in the 90 degree is going to be less whereas, the scattering would be in the forward direction is going to be more but if the material what is present inside the cell is very, very dense, then the lot of proportion of this laser beam is going to be go into the side scattering and that is how the side scattering is going to give you the idea about the density of the sample whereas the forward scattering is going to give you the idea about the size of the beat.

So, these 2 channels are being used to detect and to differentiate the different types of cells based on the size as well as the density of the molecule. So, the forward scattering is for the size and side scattering is for the density which means if a molecule is more denser, it is going to give you the more side scattering if the molecule is of bigger size, then it is actually going to give you the more of forward scattering and how this 2 properties can be used.

So, these 2 properties can be used even to distinguish the different types of cells for example, when you are processing a sample, you might actually process the you know a complicated for example, if you process the blood like so, as we see like the blood is having a size of like RBCs then you have the macrophages and then you have the monocytes and all those kinds of cells.

So, what will happen is if you process the side the blood and suppose you are processing the side scattering on this side and the forward scattering on this side, then what will happen is you are going to see the cone shape dot plot like this and where all these dot plots are actually going to say that what cell are actually corresponding to which, so, this means it these cells which are actually going to have the less amount of the side scattering and less amount of the forward scattering is actually corresponding to RBC.

Because RBCs are of very small size and RBC does not have the nucleus and organelles. So, it is his density is also very low. So that is why on the left corner, you are going to see the RBCs whereas, on the upper right corner, you are going to see the cells which are off very, very big size and there, they also have the very high density and so, what are these cells, these are the monocytes or the lymphocytes, which are being present onto the red corner of this.

So, that is how you can be able to and separate the different types of cells without even going through with the additional detectors. So, apart from these you can also have the additional detectors for the fluorescence channels for example, you can have the FL-1 you can have the FL-2 you can have the FL-3 and all these FL-3 means the flow since channel 1 flow says channel 2 and channel 3 and all these FL-1 FL-2 FL-3 are actually be dedicated channel for the specific fluorescence.

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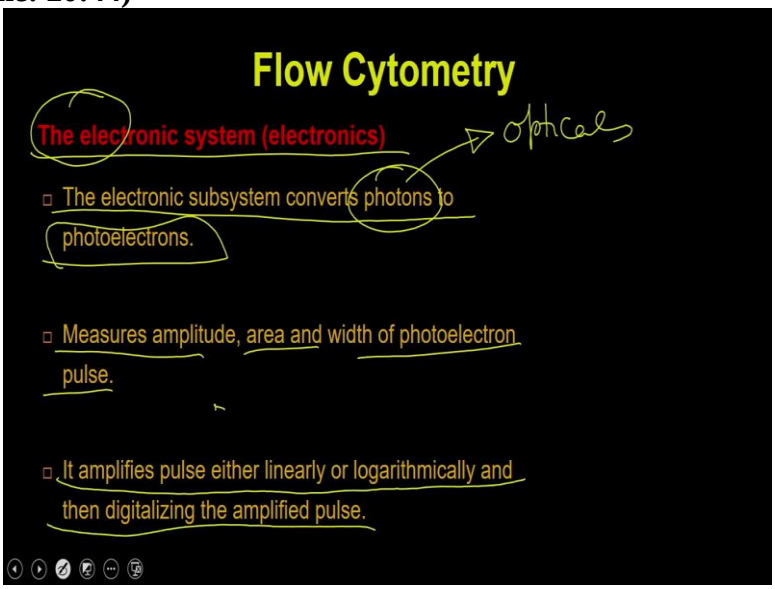

Then you have the electronic system. So in the electronic system actually converts the photons what you are going to get or what you are going to measure by the optical system into our photoelectron which means it is actually going to convert that into the electronic current and that current is going to be further convert into a readable count. So, may it actually allow you to measure the amplitude, area and the width of the photoelectron pulse.

Which means, wherever what is the height of this particular signal, what is the area of this particular signal and how many signals you are getting, that is actually going to be the purpose of having a photoelectron systems, it amplifies the pulse either linearly or logarithmically and then digitizing the amplitude pulse. So, electronic system is only to take the signal from the optical system and then it is actually going to convert that into a readable count and that is going to be visualized by the person who is doing the experiments.

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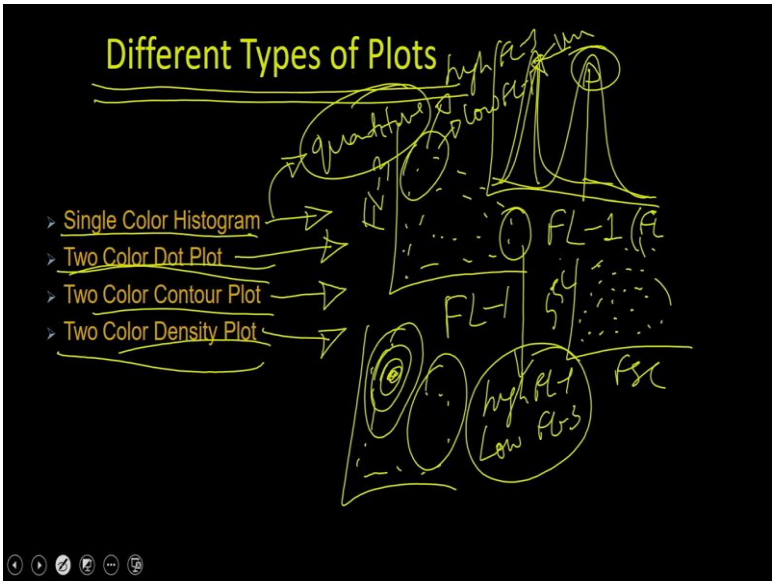

You have the different types of plot what you can use in the flow cytometer. So, you can have the single color histogram. So, what is mean by the histogram is that you can have the single property for example, you can have the FL-1, so, what will happen is you can have the histogram like this or so, for example, this is the unlabelled samples. So, this is the unlabeled sample and then this is the labelled sample.

So, you can have the single color histogram, you can have the 2 color dot plot for the dot plot means, you can have the pattern of the signal for example, on this size you probably can have the FL-3 on this side you can have the FL-1 or sometime you dot plot you can have the SSC versus FSC. So, what is going to show you it is going to show you the distribution of the different types of signal in this particular plane.

So, you can have a distribution so, what is distribution we will say that the these cells which are present on this side is actually high in FL-3 whereas, the low in FL-1 whereas, the self that is present here is actually high in FL-1 and low in FL-3. So, that is the purpose of having a 2 color dot plot. Similarly, you can have the contour plot for contour plot is actually an extension of the dot plot.

So, a dot plot is actually going to give you the dots whereas, the contour is actually going to give you contour like covering up that particular signal and then it is actually going to say that whether the signal is going up or going down. So, the contour is actually going to show you the circularized you know pattern and whereas in the center it is actually going to show

you the highest signal so that is how it is actually going to give you the contour signal and then you can have the 2 color density plots.

That density plot is also like the 2 color dot plot and it is also going to show you the density like it is very simple to similar to the contour plot but it is actually going to show you the density of that particular sample in that particular region. So, that is how you are actually have the flexibility of different types of plots whether you have the histogram plot, whether you have the dot plot.

Whether you have the contour plot or whether you have the density plot all these the purpose of all these plots are to look for the individual properties for example, you are going to use the histogram plot when you are looking at the single property varying in the different types of cells, but you can have the dot plot when you are looking for the 2 different properties and how the 2 different properties are varying in a single cell populations or in a multiple cell populations.

But on a individual cell, you can be able to ask whether this cell has the 2 properties or single properties or both properties. So that is how you actually if you have the 2 color dot plot, it actually give you the more flexibility in analyzing the samples compared to that you have the histogram plot. So, histogram plot always gives you the quantitative information or it is actually going to tell you that what is the level of this particular signal which means on this side, if you are putting the FL-1 you can have the fluorescence actually.

So, it is actually going to tell you what is the level of this fluorescence compared to this. So, it is actually going to give you the quantitative or the semi quantitative information also it is going to tell you the distribution of the samples within this particular histogram plot, but it is also it is not going to give you the information about whether that particular cell is having the second floor for or not, because it will only single color histogram whereas in the case of dot plot, it is only telling you the distribution of the 2 properties in a sample.

So, that is actually So, 2 color dot plot is always been used to qualitatively see how the 2 properties are distributing within a cell populations and then based on this, you can be able to choose a particular population and then you ask what is the level of the one signal or other

signal. So, we will going to take an few example of this and then you will understand more in detail.

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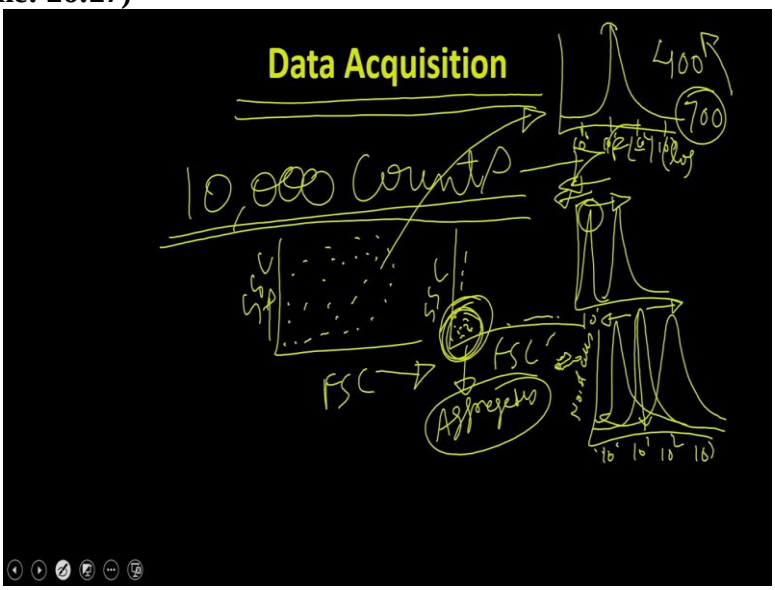

Then you have the data acquisition. So, data acquisition is very simple, you have to process your sample and then ideally, what people do is they are always collecting the 10,000 counts. So, in a flow cytometer, you do not call the number of cells you always call it as a count, because every individual cell is considered as a particle and that particle is being counted. So, what people normally do is they always collect the sample often 10,000 counts.

So, when you are going to put the sample into the flow cell or when you are going to put collect the samples to the machine, it is actually first going to allow you to collect the sample. So, what you do is first you collect the samples without even going through with the acquisition process. So, it gives you the option to suck the sample without recording it. So, what will happen is initially what you going to do is first you see whether the sample is good enough or not.

So, that is actually going to tell you have to do it with the help of the SSC versus FSC to first thing what you have to do is first you have to collect or check the quality of the sample. So, if you do the SSC versus FSC, dot plot or the 2 color dot plot for then it is actually going to give you the pattern of the sample. So, suppose this is the set pattern, you can have that 2 conditions where in one condition, I am getting this kind of pattern or the other conditions you are getting a condition like this.

So, there is no distribution or suppose this is like this. So, this is actually a sample which is well prepared, because you are getting a distribution of all the cells in the FSC versus SSC because now you can be able to see all the cell populations or the cell population present in the sample, whereas in this case, your all cells are present onto the left corners, which means either the way you have processed the sample, it is actually destroying all other samples or it is actually causing the aggregates of these cells.

So, these because they are causing the aggregates the cells are always been present at the lower left corner of the sample and then this sample is not good enough, because then you cannot do the histogram analysis and ask for any questions because you have not processed the sample properly. Now, once you have this, then you have a choice that you want to collect the dot plot or whether you want to collect the histogram plot, then after once you see that then you can ask that you want to collect the histogram plot.

So, then you can do FL-1 or FL-3 or whatever and then it is actually going to give you the sample for the FL-3. So, FL-3 or FL-1 you are always collecting in terms of the log scale. So, you can see that it is actually going to give you 10 to the power 0, 10 to power 1, 10 to power 2, 10 to power 3. Now, here also you have to always consider that when you are looking at the histogram plot and you are looking for the histogram plot of unlabelled samples.

You can have the multiple free right because the unlabelled sample is going to have a very low level of fluorescence, it is always going to be like this, but you have the flexibility in a flow cytometer that you can be able to increase the current as well as the gain of the instruments. So, what is mean by the current and the gain is that it is a virtual way of giving the more intensity to the sample.

So when you give the more intensity to sample it is eventually going to give you the more fluorescence. So that is can be modulated with the help of the laser. So if you provide more current to the laser, the laser intensity is going to go up and as a result, it is going to give you the more signal. Because you can change the current as well as the gain, you can be able to shift these peaks in both directions. So, for example, if I am using a current of 400 I can still use, I can just convert it to 700.

So, what I have done by have just increased the current to 1.5 volts. So, in that case the peak is going to be shifted onto this side, if I do the reverse the current is then the peak is going to be shifted in that this direction. Because, all the thing what you see in the flow cytometer is very relative it is always relative to what you have done for the control cells. So in ideal conditions when you are doing the data acquisitions.

And when you trying to do a histogram plot, what you prefer is that you see you started with 10 to power 0, 10 to power 1, 10 to power 2, 10 to power 3. So, what you prefer on this side, you are going to have number of cells. So, what you prefer is for unlabelled samples, it should be around 10 to power 1 and it should give you a peak. So, that; you can be able to see the change in the fluorescence of this particular labelled sample in both the direction.

For example, if I am looking at the untreated versus the treated sample, and I do not know whether the fluorescence is going to go up or go down, then in that case, if I keep the peak at 10 power 1. And suppose the peaks got shifted to this side when I am treating the sample with some kind of drug or something and the fluorescence is not going to increase then in this case, it is actually going to give you the peak on this side and suppose there will be an increase in peak then it is actually going to give you a peak on this side.

Whereas, in the other conditions when you are actually holding the peak of untreated sample very close to 10 to power 0 or less than 10 to power 0 then in that case, you will not be able to see any movement of the peak towards the left side because that time the you know left side is not existing because 10 to power 0 is the minimum scale possible in this particular type of analysers.

So, that is why it is important that you keep the peak in such a way so that you could be able to see the movement of the peak on both the sides. That is it the data acquisition has to be done on a multiple occasions like once you see the control samples and once you see the current untreated sample you have to modulate the untreated sample with the help of the current as well as the gain settings.

And then once you got the right positions then you should not change the current and the gain conditions and then you analyse your treated samples. And if you see there will be a huge shift for example, if I see that, it is actually crossing 10 to power 3 also then in that case, I can

just go back again to the untreated sample, again I will change the current and the gain in such a way so that your treated samples should come within the scale.

So, these are the things what you have to keep under consideration when you are doing the data acquisition. So, first thing you have to go for the SSC versus FSC pattern and look for whether you are getting a beautiful distribution of the different types of cells are not it should not be focused on to one region it should not be focused on here or it should not be focused here it though in the both the conditions your sample is not correct.

Because when you are processing a blood like for example, you are processing a blood, the bloods are having the cells of different sizes. So, it should give you a very nice distribution. Once you see a nice distribution then you can ask whether the sample has a signal for FL-1 or FL-2 or FL-3 whatever the fluorescent present in your cell and then you do all these modifications with the help of the current and gain.

So that when you analyse the treated sample, you should be able to get the real picture because you should have the flexibility you should provide the flexibility in the system, so that the treated sample should move towards the left or to the right.

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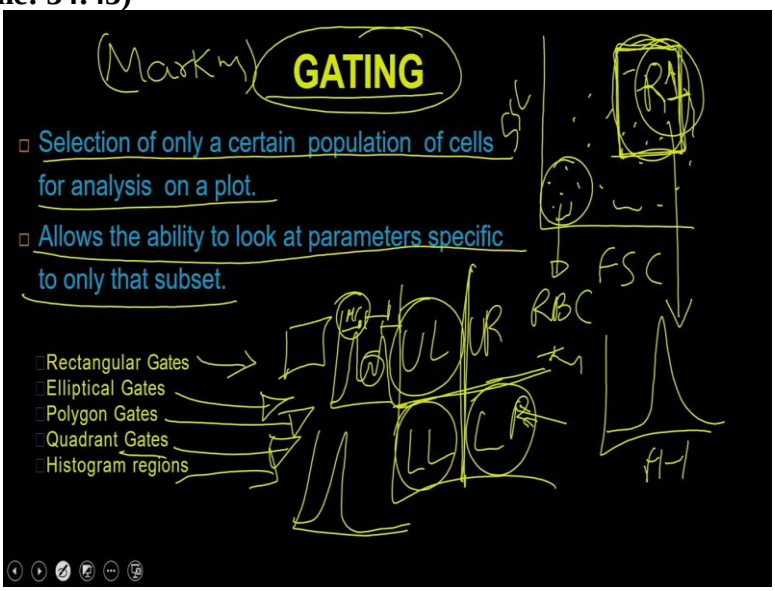

Once you have done the data acquisition, so gating is also a part of the data acquisition. So, what is mean by the gating is that see, as I told you, gating is the selection of the some of the molecules within the whole populations. So, selection of only certain population of cells for

analysis of a plot. So, it allows the ability to look at parameters specific to only that particular subset.

For example, as I said, you know, when you start the reaction first you have to do is you have to do SSC versus FSC signal. So, then what happen you are going to get a population. So, as I said, you know, these are the population which is for the, you know, neutrophils or macrophages are monocytes whereas this is a population which is for the RBC. So, what gating mean is that if I have collected the 10,000 counts and suppose, I am only interested to see the population of T cell or B cell.

So, that is not important that you use the SSC versus FSC you can use any parameter for example, you can use the FL-1 versus FL-3 and you can get a parameter. So, in a dot plot, what you are supposed to do is you have to gate a particular type of signal. So, gate is a electronic way of selecting a particular cell populations, for example, I have used the rectangular gate and I have selected this region within this FSC versus SSC.

And then what I will do is so, for example, this is a R1 gate. So, then what I can ask is, I can ask the instrument to collect the 10,000 counts not in the whole cell population, it collect the 10,000 counts only in the R1 gate. So, what it will going to do is, it is going to start collecting the 10,000 points or 10,000 counts in this R1 gate irrespective of how many samples it has collected or how many counts it has collected from the samples.

It will not stop until it is not going to have the 10,000 counts in the gate R1. That is actually and then what I will say is, I am going to say that you are going to show me the histogram for example, the FL-1 for the R1 count or the R1 gates. So, it is going to only going to show me the fluorescence of this R1 count, it is not going to give me the fluorescence of the whole cell population.

Because I am not interested to see the fluorescence of the RBCs I am not interested to see the fluorescence of the leukocytes or the T cell or B cell I am only interested to see the fluorescence of the monocytes. So, if I want to select the monocytes. I have 2 choices of selecting the monocytes either I go with the SSC versus FSC and I know where the region is corresponding to the monocytes or I can use the cell specific signalling.

For example, I can use the cell specific markers and then I can stain the samples with that particular marker to select where what is the region is corresponding to the monocytes and then I can just do a gating of that particular sample. So, I can just make a bracket with the help of the electronic tools, what is available within the software and I can just do a gating for that particular region.

So then it is not going to analyse the whole sample. It is only going to give me the flexibility of analysing the sample what is present within that gate, gate could be of different types of gates. So, depends on what kind of shape you prepare. So, gate could be a rectangular shape, it could be a elliptical shape, it could be a polygonal gate, it could be a quadrant gate, so what is quadrant gate is that it is actually going to be a checkerboard kind of thing.

So, it is actually going to give me 4 quadrants, where you have the lower left quadrants, lower right quadrants, upper right quadrants and the upper left quadrants. So, these are the 4 quadrants. So, if you have the quadrant gates, it is actually going to give you the checkerboard kind of things. So that actually is going to then your whole sample is going to be distributed into these 4 corners or 4 squares.

And it is going to give you the analysis of all these 4 squares separately. And it is going to tell you what is the cell population in the lower left corner, what is the cell population or the pattern of the population in the into the upper left corners and right corners and all that. That is very useful in some of the analysis and then anyway, we are going to see when we are going to discuss some of the experiments related to cytometers.

And then you can also have the histogram regions apart from that the gating you can also do the marking actually. So, what is mean by marking is that when you are doing the histogram analysis, what you going to do is you are actually going to have the this right or some of time you can have the even complicated peaks like you can have like this. So, in these kind of cases, what you can do is you can simply do a mark you can have the line like this.

You can have the line of the marking, so, that is a kind of a gating, but that is only for the histogram plot. So, you can just put a marking like this. So then in that case, suppose this is the M1 marker. So in that case, what we will going to what the machine is going to tell you is

it is going to tell you, what is the level of the fluorescence in the M1 marker and what is done cell population in the M2 marker.

So, you can have the different types of marker you can have the marker like for this peak, you can have the marker for this peak. So, you can have the M1, M2, M3. So, you can analyse your sample as per what is present in individual peaks, because now, what you see is, you are getting the 3 individual peaks in a histogram plot. So, if you are interested you can be able to even measure the individual fluorescence in the individual peak.

Because that is actually going to tell you whether, the peak in this marker is changing when you are treating the sample with a particular drug or particular molecules or some kind of changes what you are doing or whether the changes are happening in these because if you are looking for the average florescence the average fluorescence of untreated sample versus treated sample may not change.

But, the pattern within these peaks may change. So, that is a very very good information what you can extract after even collecting the data also. So, you can do a marker analysis and that also is going to tell you the some more information about the samples.

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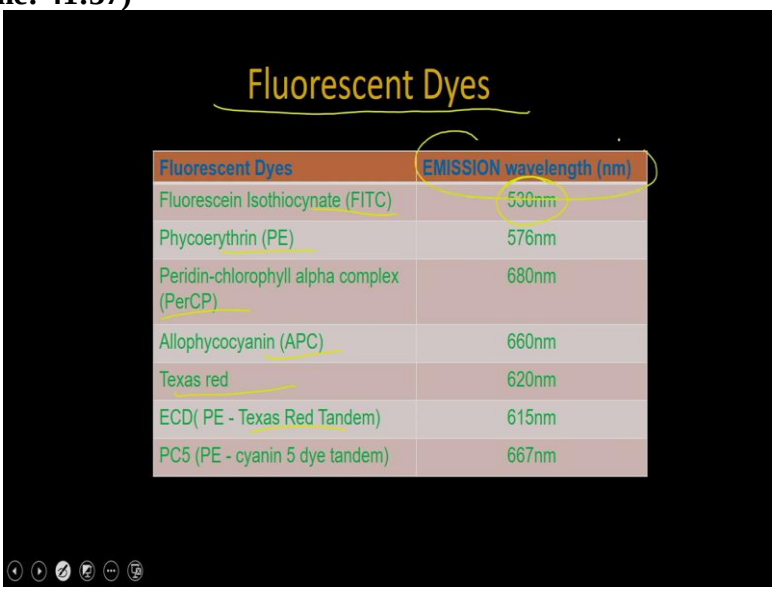

Then these are the different types of fluorescent dye you have a huge list of the fluorescent dye what you can use in the cytometers. So, what I have shown is only the popular ones like FITC, PE, PerCP, APC, Texas red and all that what you see is all these fluorescent dyes are doing a emission at a different wavelengths and that can be used event to label the sample

with the different dyes and that is how you can collect. The problem with the different types of dyes especially if you are doing 2 colour labelling is that you have to go through with the process of compensations.

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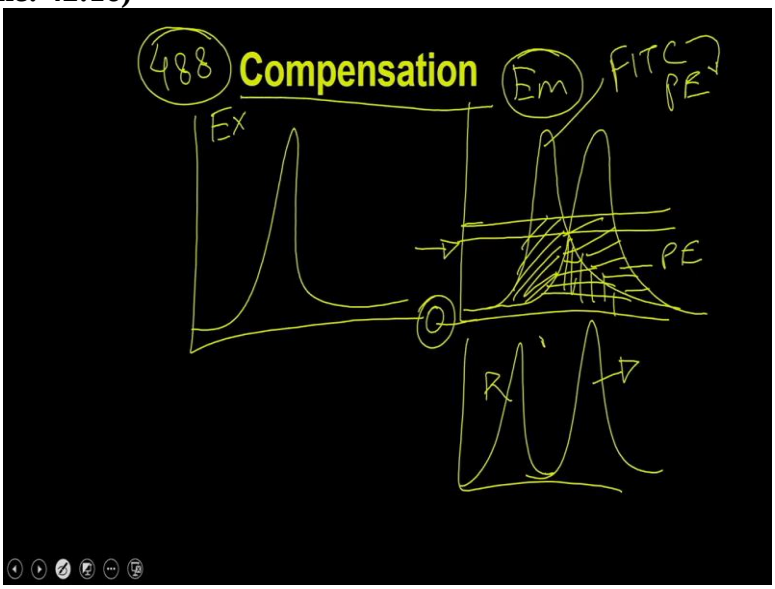

What is mean by the compensation is that you know that every dye has 2 different types of spectrum. One is called as the excitation spectra, the other one is called as the emission spectra. So, in a standard floro meter, you do not have the excitation changes. So, what we use is we always use a 488 nanometre for laser. So, what you going to do is you are going to excite everybody with the 488 nanometre.

So, that is not a problem what the problem is in the emission spectra for example, if one dye is having a emission spectra like this the other dye suppose this is a FITC. And the other dye is suppose you are using the phycoerythin or PE and the PE is having a fluorescence spectra like this, emission spectra like this. Now, if I try to see a sample and I want only interested to see the FITC or PE.

But what you do not see is the sample what is region of this right. So, you these 2 if you are using these 2 dye as a pair, what you see is there is a overlapping region of their emissions. This means the sample FITC signal will bleed through into the PE signal and the PE signal is bleed through into the FITC signal which means there is not going to have any purity of the sample.

Ideally what I should have the signal like this I should have a fluorescent signal of FITC like this and the PE for this. In that case this is ideal condition where there will be no overlap of these 2 dyes. So, whatever I see is actually a pure filled cell. So; if I see a signal which is only having the FITC signal, it is actually a pure signal, but in this kind of situation where you have the overlap of the 2 different types of dye emissions, this is the region which is actually going to create trouble.

So, what I have to do is I have to collect the sample and I have to cut down the sections I have to cut down the data in such a way so that if I suppose this is a background. So, it is this is the 0 fluorescence. So, if I increase the level of my background, and if I make it slightly bigger, for example, if I go up to this, then I am achieving this particular kind of conditions. But what is happening when you are going through this process of cutting the data.

You are actually losing the data, for example, you are losing this much from the FITC and you are losing this much from the PE which means these are the things what you have to do when you are using the 2 different dyes, which are having the overlapping emission spectra and that is the process is called as compensations. So, when you use the 2 dyes, the software is going to do the analysis and it is going to tell you that how much is the background signal you have to uplift so that you will be able to overcome the compensation.

Or in some cases, it is actually going to do the integration of these 2 signals, and it will tell you that if you see a FITC signal, it is going to be contaminated with the 15% of the PE signal, if you see a PE signal, it is going to be contaminated with the 25% or 22% of the FITC signal. So, that is a kind of a subtraction it has going to put when you are going to go through with the analysis process and that is how the compensation is going to help you to overcome this kind of situations. And that it is always been a inbuilt into the software it is going to allow you to go with the compensation process.

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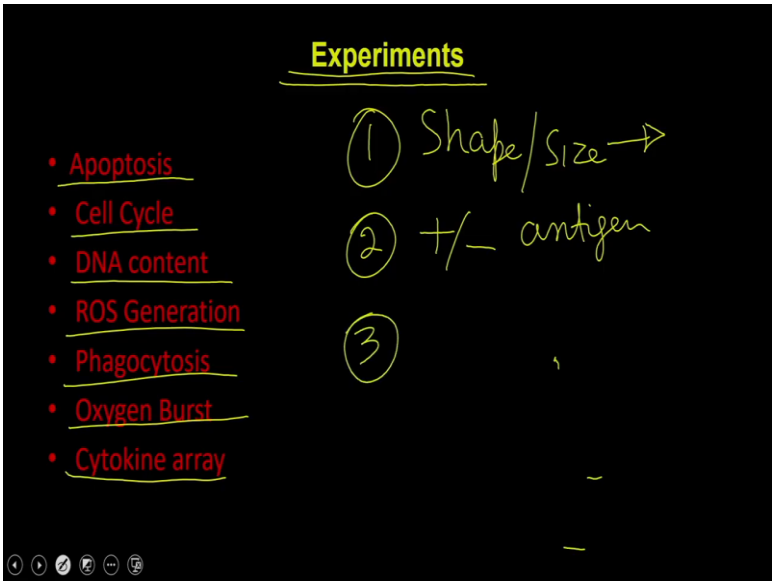

Now, let us discuss about the different types of experiment what you can do with the help of the flow cytometry. So, in the flow cytometry, you can do the apoptosis, cell cycle, DNA content, ROS generation, phagocytosis oxygen burst and the cytokine array. Ideally, the flow cytometry can be used for 2 or 3 different types of experiments, what kind of experiment you can do, you can do an experiment where there will be a change in shape or size.

So, you if there will be any change in shape or size, you can use the flow cytometry because it is having a FSC versus SSC module, so, that can be used to detect whether there will be a change in shape and size. Number 2, you can actually do fluorescence or cell cytometer if there will be a appearance or the disappearance of a antigen or the molecule from the cell surface, because then you can do imaging or then you can do the staining of that particular sample.

Number 3, you can also use the cytometer when there will be a change in the cellular content as well. So, for example, you can or there will be appearance of a particular object. So, these are the 3 different criteria, which are mainly been people used to design the experiment or to consider the cytometer as a potential to answer the questions, these are the examples what we are going to discuss, but these are not the only thing what we can do with the help of the flow cytometer we can do even further up.

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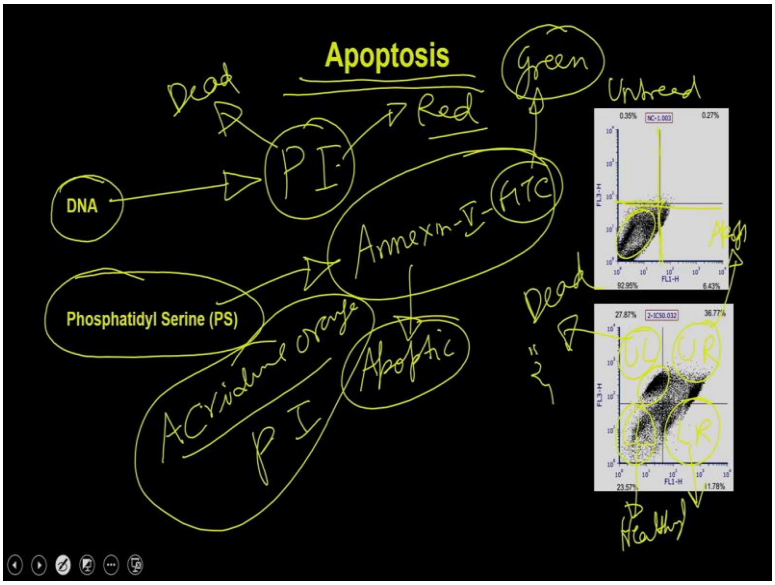

So, as far as the apoptosis is concerned about apoptosis is a process which is also called as the program cell death and the program cell death is always been resulted into the degradation of the DNA as well as the externalization of the phosphatidyl serine onto the cell surface. This means, it this is the kind of examples where you are actually going to see a change inside the cell as well as the change outside the cell.

So, what you can do is you can actually stain the DNA with a DNA dye for example, you can use the propidium iodide or you can stain the some reagents, which actually going to bind to the phosphatidyl serine. So, for example, you can use the annexin. So, annexin 5 which is actually labelled with FITC. So, you are actually can use the annexin 5, which is labelled with FITC and annexin 5 has a very, very high specificity for the phosphatidyl serine, which is been present onto the external surface of the cell surface.

And you can also use the propidium iodide. So, propidium iodide always gives the red signal, the FITC is going to give you the green signal. So, if you stay in the cells with the red, the PI as well as the green signal, the PI is going to label the dead cells, or whereas the FITC is going to all the annexin is going to label the apoptosis cell. And that is how you can be able to analyze apart from this you can also use the another combination, which is also called as the cell combination like acridine orange versus the PI so propidium iodide.

So, if you use the these 2 dye as well, you can still be able to detect the signal because what happened is the acridine orange is a dye which enters into the cell because you know apart from there will be externalization of PS onto the cell surface. The cell membrane

permeability is also been compromised in the case of apoptosis. So if there will be any compromised cell permeability, it is going to allow the moment of these dyes.

So acridine orange is a specific dye, which always goes inside the cell and it is going to label the DNA only in the cell is compromised and the cell membrane is going to be compromised, whereas the PI is going to label the cells irrespective of this. So this is the data what you see So acridine orange and PI, if you stain, and this is what I was talking about the checkerboard analysis, so, what you do is when you stain the cells, you are going to have the 2 samples, one is untreated sample, the other one is treated samples. So then what you do is you just do a checkerboard analysis.

So, these are the healthy cells. So, what you do is you do a checkerboard analysis and only cover the healthy cells, and then you are going to analyze the treated samples. So, what you would see is that the upper left corner, upper right corner, and the lower left corner and the lower right corner, now these are the cells which are actually going to have only the propidium iodide.

So, these are the cells which are actually the dead cells these are the cells which are having the both the dye in high concentrations. So, these are the apoptotic cells, these are the also apoptotic cells, and these are the healthy cells. So, that is how you can be able to use the staining techniques with the different types of dyes. And you can use the checkerboard analysis with the help of the getting the and that is actually going to tell you, what is the cell population within the sample is apoptotic.

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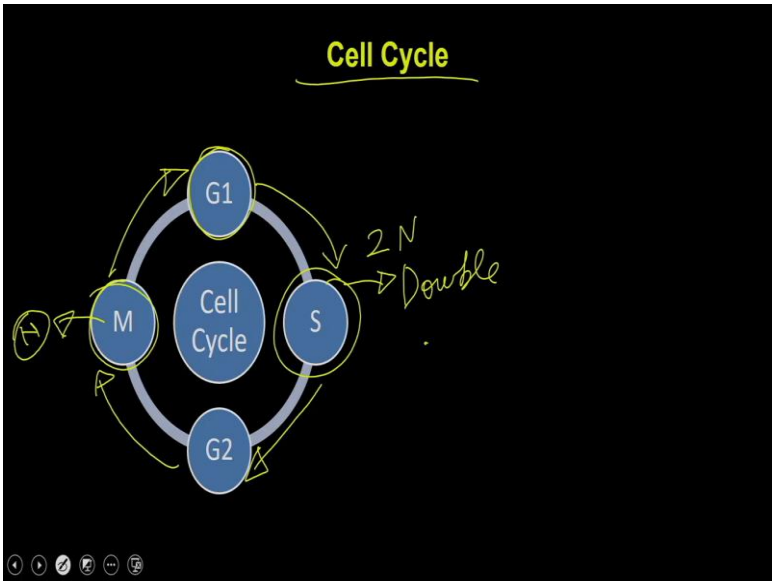

Then we can talk about the cell cycle. So, you know that the cell is going through a different cell cycle and that is actually going to happen that it starts from the M. So, in the M, then it is going through a preparative stage it is making a G 1 then it is entering into the S phase at this stage that DNA is going to be double and then it is entering into the G 2 for DNA still double and then at this stage the DNA is going to be cell is going to be divided and that is how you DNA is going to be normal.

So, you can imagine data if I am starting with the N amount of DNA, it is going to be 2 N at this moment and then again it reaches to the N. So, if I want to study the cell cycle, what I can do is I can just stain the cells with the propidium iodide and I look for the content of DNA.

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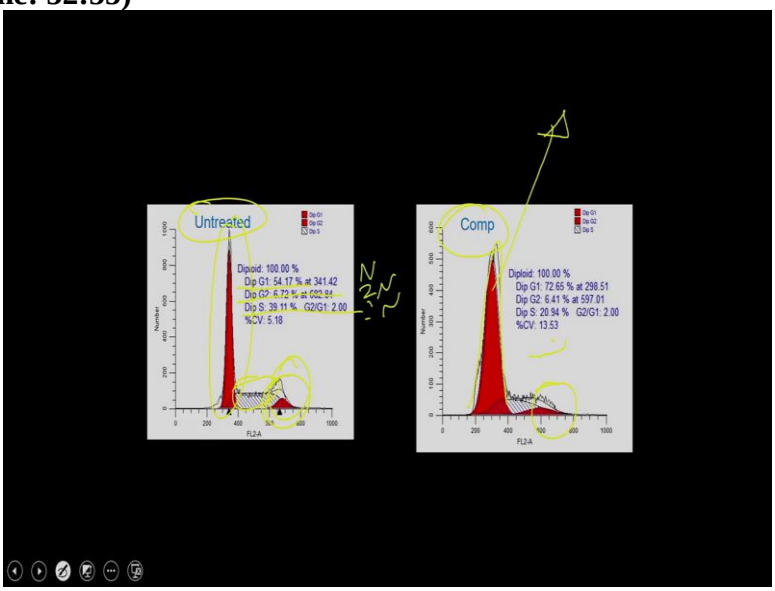

So, ideally what will happen ideally in the G 1 you might have seen that it is actually the N G 2 also it is 2 N and the S it is actually 2 N. So, what that is what happened in the this is the your G 1 phase, this is your G 2 phase and in between what you see is actually the S phase. So, once you characterize the G 1 S and M G 2 phase, it is actually going to give you it is going to allow you to study the D cell cycle.

So, what you can see now, here, this is the untreated sample, this is the treated sample, and what you will see is there will be a disappearance of G 2 M phase, which is actually this phase and there is a increase in the G 1 phase, which means the cells are actually not going beyond the G 1 phase which means they are not entering into the S phase.

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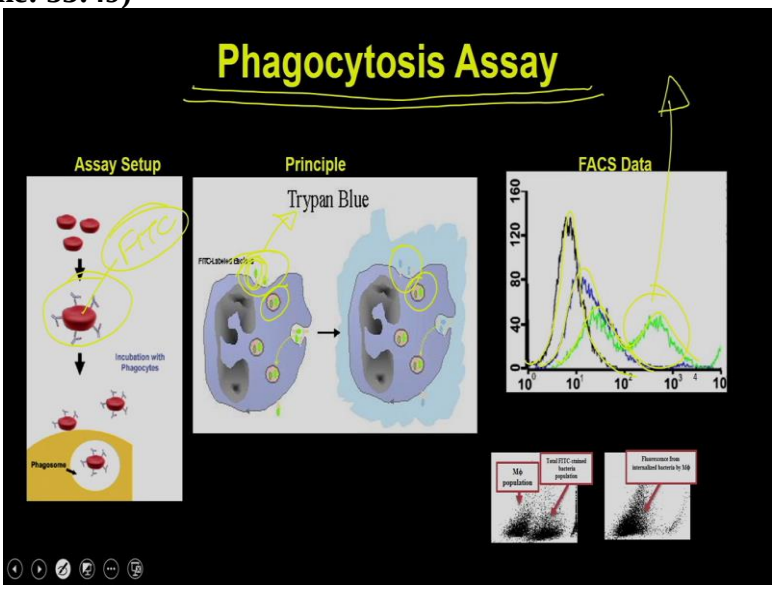

Then you have the phagocytosis assay. So, you know the phagocytosis where if you add a particle to the macrophages or any phagocytic cells, they actually engulf that particles. So, in this particular essay, what we are doing is they are just simply using the RBCs and they are labelling it with antibodies. And the basic principle is that you can actually label these RBCs with a dye which is called as the FITC.

So, what happened is, once the FITC are present outside you can have 2 conditions where the particle is present outside or particle is present inside. So, when you are actually having a particle outside it is very sensitive for the trypan blue because the trypan blue is a very good quencher for the FITC. So it is not going to allow the FITC to give you the signal. And that is how you can be able to discriminate what is the amount of the particle present outside and what is the particle present inside.

Because you know, that principle in trypan blue will not enter into the healthy cells that side this signal is going to be remained intact whereas the signal is going to be quenched. So this is what exactly going to happen. These are the normal cells as I said you know you always have to take the healthy cells first. And then these are the untreated cells and these are the treated cells. So what you see is that this is the cell population which is actually has taken up the trypan blue and that is how it is actually showing you the fluorescent signal.

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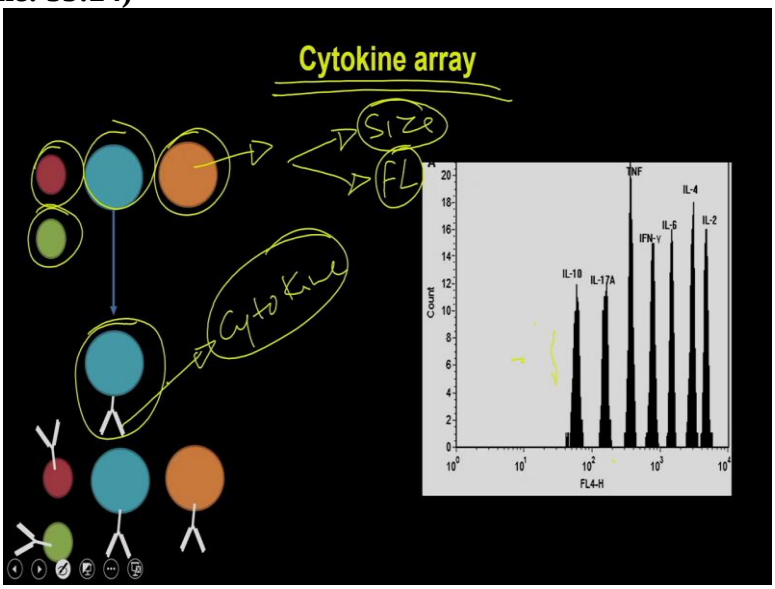

Then we have the cytokine array so because of floors, the facts of a flow cytometer is you has the potential of analyzing the different sizes of the objects as well as it is actually having the flexibility of analyzing different types of fluorescence, the people have also designed the cytokine arrays, which actually can allow you to measure different cytokines in a different in a particular given samples.

So, what you people are doing is they are taking the beats of different sizes as well as the different fluorophores. So, these beats are actually having the different fluorophores mostly these beats are the quantum dots what they are using have different sizes. So, you can have the 1 nanometer 10 nanometer 100 nanometers. So, all those kinds of sizes of the quantum dots, which are actually going to give you the different types of fluorescence, and now, what you going to do is you tag these dyes or the beats with the antibody.

And these antibodies are going to be specific for a particular type of cytokines. Now, what you are going to do is you are going to develop all these beats and then you mix them. So, you have the different sizes beats, you have the different flow for beats and then all these

different sizes as well as the different fluorophores containing beads are tagged with different antibodies.

So, you can have the antibodies for TNF alpha, you can have the antibodies for aisle 1 and so on. So, now what you going to do is you analyze you incubate these beads along with the cell supernatant or the cytosol where you are interested to measure the cytokines. So what will happen, these antibodies are going to bind their respective cytokines. And now what you are going to do is you are going to analyze this into a fax machine.

So, what the fax is going to do is it is going to separate these cells based on the size as well as based on the fluorescence. Now, what will happen is that if they are actually going to have the different types of bindings, so, once you added it, you can have the separation based on the size as well as based on the fluorescence and height of these beads are actually going to tell you the concentration of that particular cytokine present in the cell supernatant.

So, as you can see here, this is the 1 beat which is actually going to give you a histogram and that is corresponding to the IL 10. Similarly, you have the beats which are corresponding to IL 17. Then these are the beats which are for TNF alpha. So, because they are actually making the individual histograms, you can be able to very precisely say what is the level of TNF alpha, what is the level of IL 16 and all that.

So, you can be actually having the flexibility of measuring all the cytokines in a given sample in a single shot, both for the control as well as for the t test samples. So, this is all about the flow cytometer and how you can what are the different components are presenting the full cytometer and how you can be able to modulate the conditions. So, that you can be able to analyze your samples and with this I would like to conclude my lecture here. Thank you.