Immunology
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Lecture No -30
Flow Cytometry

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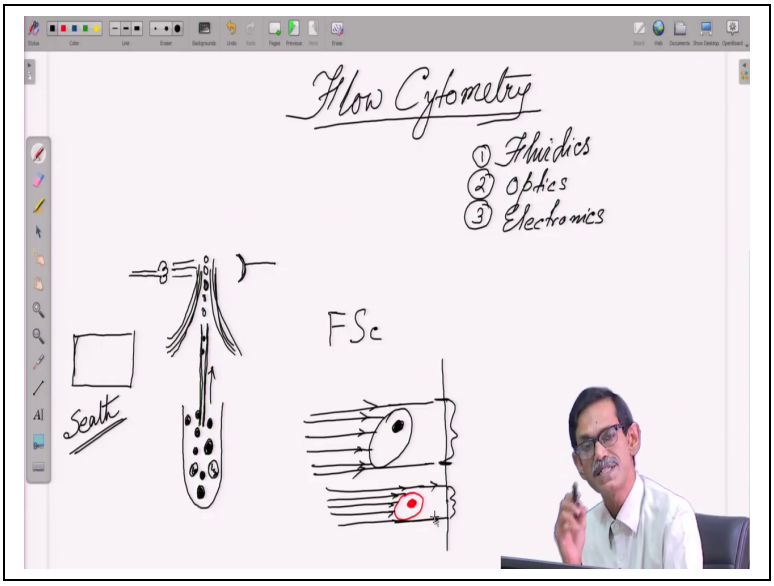

So welcome to today's lecture as we have discussed in the last lecture we are going to discuss today about flow cytometry. So flow cytometry so from the name you can understand that there are some flows of liquid right and cyto means cell and metry means measurement. So cell in suspension or in fluid we are going to measure the some parameters of cell that is why the techniques is called flow cytometry.

And the instrument we are going to use is flow cytometer. So this machine is also very commonly known as FACS fluorescence activated cell sorter but this is not the name of the general name of the machine FACS is the name of a company. If the vector dictionary named it so what actually doing it by this as we have discussed in the last class if I, stain a cell with a fluorescence dye that see another microscope we can see few cells may be hundred if you give lot of time.

But if you want to measure in population study like how what is happening in ten thousand cells or a million cells then manual it is not possible. So this machine is actually going to help you how many cells are what color and that color depends on what you want to do? So by this machine what we can do is we can measure the size or we can differentiate a mixture of cells of different size and their complexity and definitely the protein expression.

If we would like to determine any protein that we can use the same immunostaining method by fluorescence labeled antibody and we can measure it. So there are 3 basic components in this machine one is definitely we have to handle some liquid so that is fluid x so that needs a pump which will suck the cell and do the I mean pass the cell through the detector. And second is we just need like any other spectrophotometer or any measurement because we are going to use the light to see the fluorescence and other parameter we need optics.

And third component is just to measure and to digitize the information that we are getting is electronics. So, this is 3 components are very distinctly doing their own job. So fluidics, optics and electronics what is there? The basic unit is so there is a light source. So light is going in the path of the light if there is a cell, suppose this is a nucleus and this light will interact with this cell and there is a detector this is a common what I am saying there is a detector here which will detect the signal and give it.

So cell is going to come here by fluidics optics is doing its job and the detector which is linked with a photo multiplier tube PMT photo multiplier tube which can amplify the signal if you need it and then if this signal will go and electronics part will get the information digitize the information ok. So this machine actually is completely computer operated. And so you do not have to do anything with the machine. Machine has very simple button there are only 6 buttons one 3 buttons actually controlling the flow how fast the cell will move.

So there are 3, one is slow, medium and fast another machine another button is run so when you are ready you press the run it will do its own job. So when the run is complete third thing is standby it will automatically go to standby but if you want to do it forcefully like something is wrong or you do not want anymore you want to stop the calculation or the measurement you

press the standby. And the 6th one is called prime that is normally we do not use just to clean the machine or the pipe.

So what is there actually? So let me go stepwise so if you take suppose this is a tube and there are cells there are cells. So if you take blood what is there blood is composed of varieties of cells different size different you know blood has platelet blood, has RBC, blood has lymphocyte, monocytes or macrophage, neutrophil, basophil, eustonophil all we have discussed. So it has varieties of cell here.

I hope many of you know or all of you know the bacterial cell counter or the culture counter the electronic counter what is there. In bacterial counter the idea is very simple so you know I mean you take bacteria in a tube so there are a lot of bacteria here. So, and if you have a capillary here; so and then you suck the cell. So what will happen back will move from solution through this pipe right and if there is a light source here.

And what is going to happen is suppose light and there is a photo multiplier tube I am taking the simplest way of measurement whatever it is. As soon as one cell will come here there will be a shadow and that shadow will be counted and that is very fast you do not have to count by in hemocytometer and you do not have to test just put that machine run it. So each cell will go each shadow will be counted and finally you can say after certain time or in 1 ml or 10 microliter or 100 microliter how many cells are there.

So that is how bacterial counter works it is very I mean this idea if you expand and do lot of good I mean more activity then this FACS will come it also you take the tube you have a very thin capillary. So there is a suction cell will move through this capillary and move through this capillary this way what happened there is a tank. In this tank there are certain fluid this is called seath fluid which is mostly I mean this is I mean commercial trade secret it is not known exactly normally the company is applying it but it is generally the phosphate buffered saline.

So that cell you have to maintain the osmolarity so cells should not lies. So from that source what happens there is a pipeline I am not going to I mean detail of this drawing what is

happening the I mean up to certain level there is a capillary what you can see and after that inside the machine through which cell will move ok upward after that within the machine there is a flow of liquid. So automatically from both side if the liquid flow like this.

So there will be jet and through this cell will move blank I mean there is no pipe or anything. So while this cell is going light is coming from this side. So this case the light is laser. So light will pass and there is detector. That detector will detect what this detector will needed. First thing what I will say is it is called forward scattering and in short it is called FSc. So what is happening, so suppose this is the cell this is nucleus light is coming like this way.

So now if you see these cell will block some light and this will go if I make it over simple just to make you understand easy way or easier way. So what I am saying so then this there will be so suppose there is a plate here what will happen there will be a shadow is this size right. So if there is another cell which is say small which is small. So same cell I mean similar cell but this is a small cell same way and if the light come suppose this cell is not there this cell is there so I am drawing parallely but it will happen one by one. So again same source will give the light what will happen the shadow size will be smaller than this.

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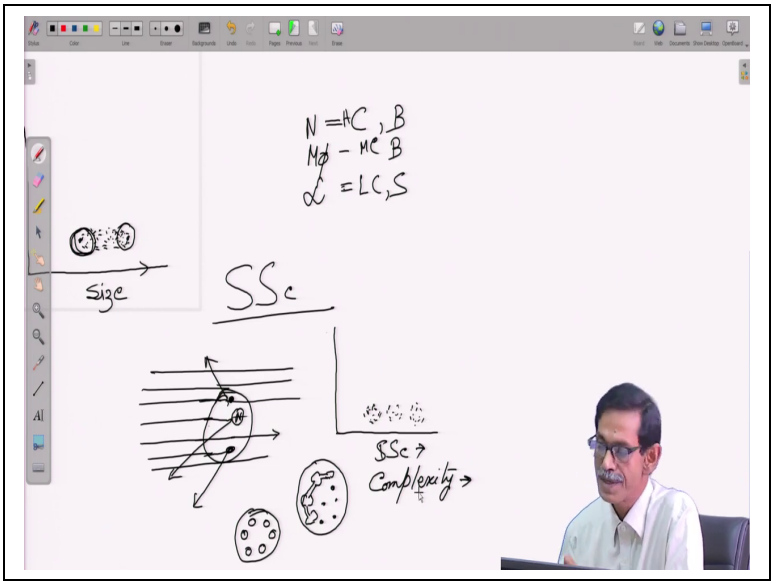

So if there is a detector which can detect the area of shadow what will happen and I am going to plot them I am going to plot according to size. So what you will see; you would not see the cell.

What you will see you will see a graphical representation of this. If this is the size and this is arbitrary unit if this is the size each cell will pass that capillary you will get one dot. Suppose the size is increasing this direction.

So what will happen suppose there is a big cell you will get a dot here. If there is a small cell you will get a dot here. So suppose this is the smallest cell and this is the biggest cell. So all the cells will be either this size or in between this size as a result so if you run 10000 cells you will get 10000 dot where the dot will be? So dot will be like this each cell will go you will see a dot. So that means in this region you will get lot of dots. So this region will be the smallest one this region will be the biggest one, clear.

So forward scattering wise each dot is going to tell you how many cells are big how many cells are small how many cells are in the medium size or in between small and big. Same way there is another call another parameter we can measure this is called this is called forward side scattering. This light will scatter sidewise side scattering. What is going to happen if this is the cell and this is say nucleus again light will come same direction same I mean ok but what will happen some light which is interacting with the cell will go.

And if there is a particle inside that will deflect it will deflect this way right. If there is no particle it will go away and if there is some particle here there are a lot of particle it may be nucleus and it will deflect in different direction. So all this side wise deflection of the light will be measured or sideways scattered light will be measured. So which cell will scatter sideways most if they have more particles inside.

If cell does not have more particle most of the light will go. So if you remember the blood what is there are neutrophil, if you remember the new if you remember the neutrophil sorry if you remember the neutrophil neutral is complicated nucleus it has lobe like structure right. You will see much better picture in book and there are lot of particle inside and if you remember the mast cell there are lot of histamine granule inside.

So there are lot of particle if you see lymphocyte there are very little particle inside right macrophage bigger but less particle so now if if I see neutrophil, neutrophil is what? neutrophil is particle wise if I say that this inside is complex the granularity is much more so neutrophil is complex as well as big. Macrophage is not much complex medium complexity but big. Lymphocyte, lymphocyte is small sorry small should not be here lymphocyte is not complex at all.

And small so what is the combination so it is not medium complexity complex medium complexity or I can say high complexity medium complexity or low or no complexity low complexity. So high complexity is a neutrophil medium complexity is macrophage low complexity is lymphocyte. Size they are big, this is big, this is small. Now if I make a plot according to this so again I mean I am coming back to this side scattering. So if I plot this and this is the complexity or granularity.

So this is side scattering. So side scattering is increasing means complexity is increasing. So, more complex cell is neutrophil. So neutrophil will come here macrophage will come here and lymphocyte will come here. So you will get very 3 distinct population. All dots means each cell. Normally we call it event. So 3 different complexity high complex will be here medium will be here low will be here because it is distributed according to the internal granularity or the complexity.

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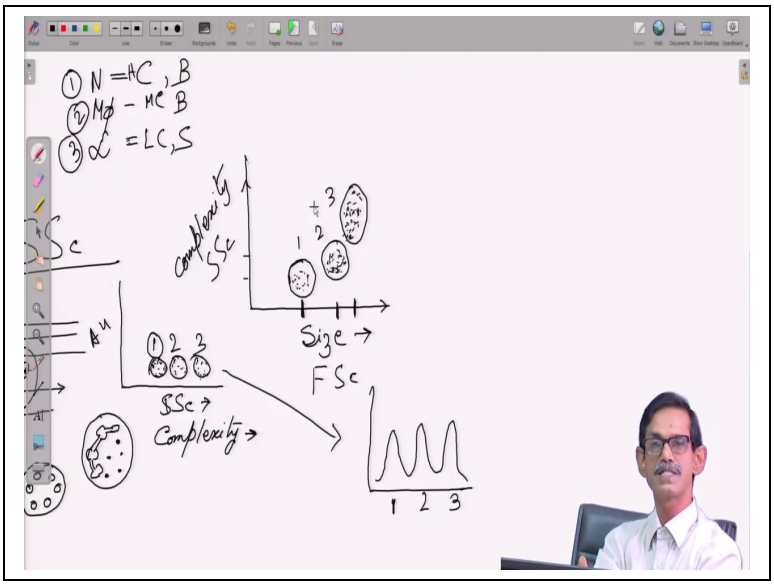

Now if I draw the same if I draw the i and one good thing I should mention that this particular machine when one cell is going upward all the parameters are going to be measured because you would not get this cell you back. So if you remember this part what is happening cell will go cell will go through this liquid from this tank and it will be collected in another tank which is waste tank.

Waste tank means that all cell will be mixed you cannot get that back it will be too diluted you cannot use it that is one disadvantage of this machine. But once it is going so it will it has several source of light and it has a several detectors. So it has a detector for SSC it has a detector for FSC ok I will come some more detector. So for the timing we have two detectors so one cell will pass here SSC will be measured.

Same cell will go here FSC will be measured. So size complexity will be measured of the same cell simultaneously. So, data is stored in the computer clear. So now if we data is already there so now if we plot suppose this is size, size means forward scattering and this is the complexity. Complexity means side scattering so if I ask the computer to plot this what is going to happen I am going to plot this because cell has this.

I am not considering the RBC and platelets because you can remove it platelets are too small because when you measure something your machine has certain limit it cannot make measure

something bigger than a limit and it cannot measure if anything too small than its limit. But in blood cell lymphocyte to neutrophil they can measure very nicely. So what is going to happen? Size wise if I distribute what will come first it will it will give a population here.

So this is what so if I see this is less complex smaller in size less complex because this is here complexity is increasing this way and smaller size. Then medium size is what medium size is macrophage. So macrophage will come here and it has a it is little big. So it is come here and complexity is medium. So it is slight more complex if this is the complexity scale, so it is slightly more complex than lymphocyte right. Now if I go for neutrophil it is bigger not as big I mean too big but it is very close to macrophage but bigger than macrophage but highly complex.

So it will be slightly bigger than macro is here this is lymphocyte if it is slightly bigger it will come here but is highly complex. So it will come here and there are some in between complexity it will come here so that way if you just run the blood cell automatically this machine will distribute according to their complexity and size. So you do not have to do anything extra you take the particular cell you want to measure suspend it should be single cell suspension.

If it is a clump you cannot do that and you can instruct the machine through computer I need count of 10000 cells or I need count of one million cells. So that I am not going experimental detail because there is no point you just understand the principle that should be enough for this. So this is the distribution plot this is called dot plot. But if I want to plot a histogram plot of this suppose this one this 3 population 1, 2, 3 what is this? This is suppose, this is 1, this is 2 and this is 3 so this 1, 2, 3 will come.

1, 2 and 3 in this plot where only complexity and this is arbitrary unit is measuring. So now same plot we can also ask the machine to make a histogram how it will come? It will come like that area is going to give you that area is going to tell you that what this peak are so this is again this is same thing this is one that means this is population is neutrophil as lymphocyte this is macrophage and this is neutrophil.

So you can have dot plot you can have histogram plot you can have um contour plot density plot whatever kind of plot you want you just have to click in the right place in the computer software or the program you do not have to do. But sale will go and all data is stored. Now it is up to you which way you want to come count. And another good part of this machine or the software or the program is if you just get this region or select this region by mouse and ask suppose this is 1, 2 and 3 and ask that give me statistics.

It will immediately tell you how many cells are there in the region you selected for one how many region how many cells are there in region 2 how many cells are there in region 3. So you do not have to do anything you just take the cell in a tube fit into it press run it will go automatically everything will be done you just get the data from computer and do it I mean analyze it depending on what you want to do.

But this is not as simple as this definitely you need to calibrate the machine you have to know the software.

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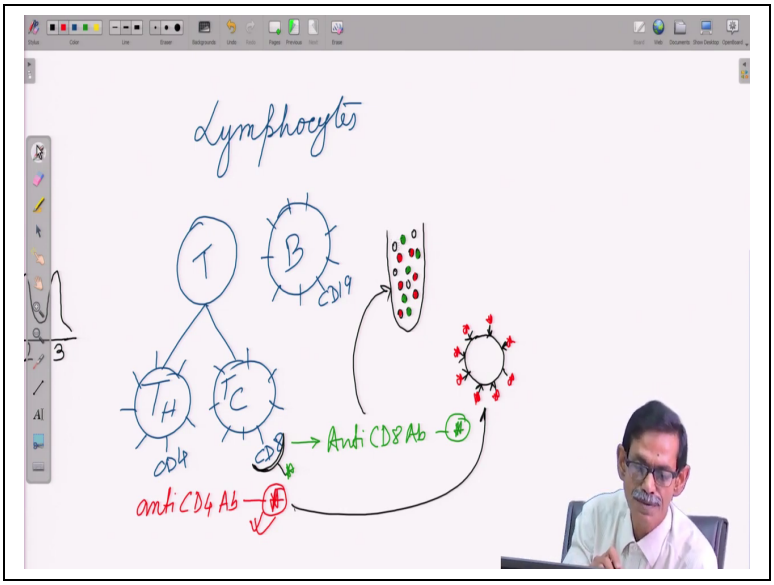

So these part is without fluorescence same way same way what we can do is suppose we have in blood in lymphocyte say in lymphocytes what we have we have a general population of T cell we have B cell right. So let me consider only this there are many I mean there are n cases also in T cell also we have T helper cells we have cytotoxic T cell and we know what is their marker T

helper cell has what CD 4 not 1 they have many CD 4. Similarly cytotoxic T cell is what cytotoxic T cell has CD 8.

What I did not tell you B cell has another kind of marker it is CD 19. So it is CD 19 is there, so these see all are CD 8. So now the technique we learned before is what immunostaining. What we can buy is we can buy anti CD 4 antibody I can buy from market which is labeled with a red fluorescence. Same way I can buy antibody CD 8 antibody which is green level. So now if I take blood where all the lymphocytes are there or if I can take cells from spleen which is loaded with B cell and T cell not blood say I isolate the spleen.

And from spleen I isolate the cells so that will be loaded with B cell and T cell so all B cell and T cell will be looking same so you cannot distinguish what which one is what, so it is mixture. And there are lot of other cells also all are say you isolate the lymphocyte and k cellular also there. So now here in this cell mixture if you add this the antibody what will happen antibody will go and bind here which is green.

So under microscope of fluorescence microscope what we will see is some cell you will see green after that you wash the extra color then you add this one in the same sample. What will happen all the CD 4 cell will be red because if this is the cell. If suppose this is the cell what will happen all antibody will go and bind right all antibody will go and bind on the receptor because this is antigen. And each antibody suppose I am talking this one if antibody is red antibody is red because you purchase red colour.

So if you see this cell under microscope cell will be look like this red. So some cell will be red some cell will be green some cell will be colourless, clear.

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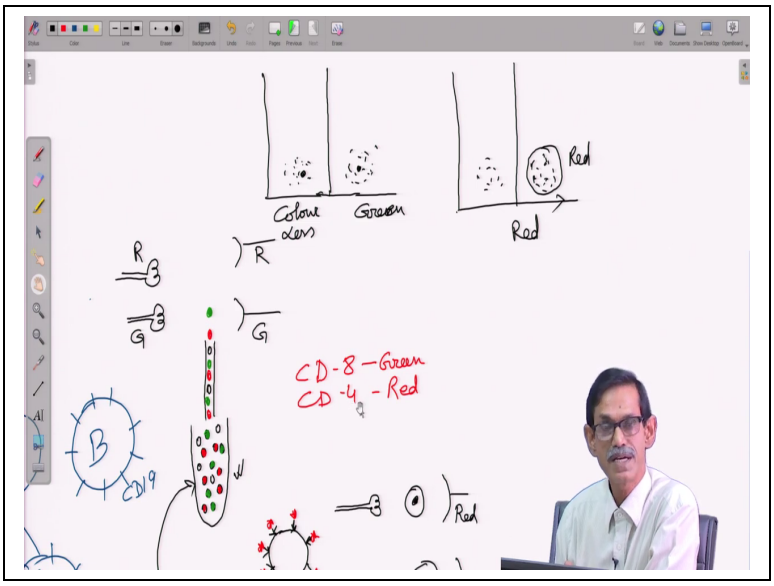

Now you remember this so what we have to remember we have to remember that CD 8 is green CD 8 is green CD 4 is red. We have to remember that okay so when I am taking talking about the light source and detector. So what is there here also in that laser there are specific laser which can activate the red fluorescence. So there and cell is here this is a cell and there is a detector which can detect red fluorescence.

Similarly another light source is there which can activate the green fluorescence. So cell is there if it is green detector is green. So in this case if this is suppose this is the light source this is for red this is the light source which is green and you have cell here. Cell from where it is coming it is coming from here it is coming from here or if I go like this if I if the cell is moving this way and the data in the light source are here this is red this is green detector are here.

So this is green detector this is red detector, so when the green cell will go here when the green cell will come here same way we plot what is the plot. So we here we will divide this plot here, here we will divide the plot this way anything beyond this line is green and this is colorless so when one green cell will come one dot will come here when red cell will come here.

So this will come here so any green cell is going to come here all other cell will come here so same way you will get lot of dot here for colorless cell and lot of dot here for green. Same thing we can get same thing we can get for red. So when the red cell will go so cell will go one by one

right so one cell will red then red then some colorless cell will come in between then some green cell will come.

So it is random so when this will pass one red cell will go one dot will come in the red one dot will come in the red zone and one green cell will come green one dot will come in the green zone. So you can see simultaneously so we will get lot of red cell here and lot of colorless l here. So in that way if you mix the cell and measure it and ask for the statistics you what we can get we can tell how many red cells are here.

What is the red cell what is red cell red cell means CD 4 right. So we can easily tell how many CD 4 cells are there. Same way I can tell how many green cells are there what is the; what are the green cells green cells is CD 8. So in a population within say 30 seconds to 1 minute we can tell how many what is the percentage because total cell I know. I can tell you how what is the percentage of CD 4 what is the percentage of CD 8 that way you can count any kind of blood cells if you know the marker.

So that is actually facts or flow cytometry what we can measure also this way like this. So red is this total is this or vice versa. So this is the flow cytometry and in next lecture or next class whenever we need flow cytometry then you can understand a little more little better way than what you understand. And I will suggest you to go and read the book because this is you do not have to be master in flow cytometry but you should understand what is this. Till then see you in the next class.