Immunology
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Lecture No -29
Tools and Techniques (Contd)

So in last class we are talking about Western blotting and in last class saying that immuno localization. Why immuno localization what does it mean we know localization actually means here whatever protein we tested like presence antigen and their molecular weight the protein is isolated from the cell or the system. So you purify the protein or extract the protein from cell and then you detect whether it is there how much is there and you use antibody for that.

Now if I would like to see that any particular protein where it is located inside the cell I am telling that this protein is present in the cytoplasm and you are believing it but if I would like to but in see you remember I told you that one technique is there by which you can see or visualize that how many MHC molecules are there in the surface. And if you give bacteria to macrophage and incubate for a certain time then you will see the number of MHC molecule is increasing on the surface of that particular macro phage cell.

You cannot do it with a Eliza or Western right you can estimate yeah total amount of protein is increasing so the in case of eliza the intensity or the OD will increase you can say but whether it is in cytoplasm or in the endosome or in the membrane you cannot say. But if I tell you something by which you can directly tell see this is cell you see more number of MHC is there it is much better and more convincing.

And this technique is all immuno localization which is again a very important technique in cell biology.

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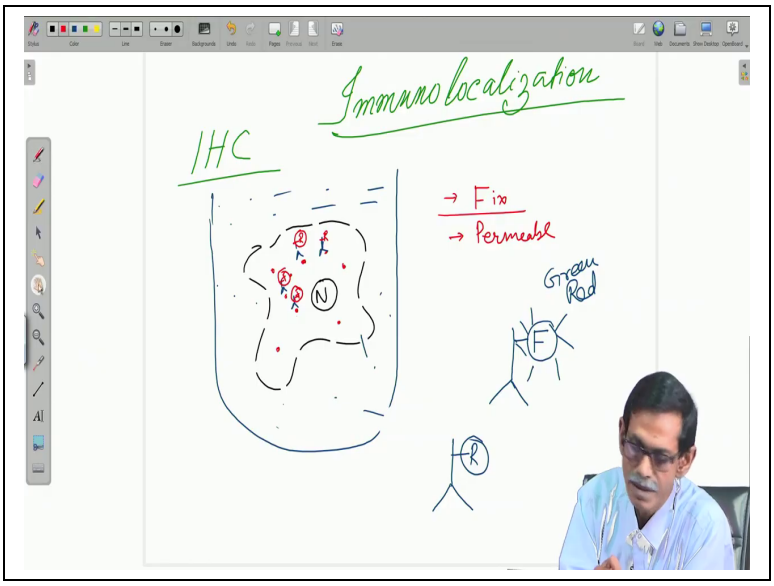

And this is immuno localization it is also called immunohisto chemistry IHC because when we localize cell in the tissue it is immunohisto chemistry histo means tissue right. So either way principle is same what we do is suppose this is a cell, this is the nucleus and I would like to see any some protein that is I am interested say for example before I may see let me start with something else suppose one protein is staying inside the cell some protein are staying inside the cell and they are here.

But in cytoplasm you cannot identify suppose this is in the cytoplasm protein means cytoplasm you cannot say that these protein you are seeing like this everything is mixed up ecology ER so many organelles are there. And cytoplasm material there are 1000 of proteins are there whether your protein is located where you cannot see but here what we can do is how we do the immuno localization we are making the membrane porous. Just first what we do is first we do normally we fix the cell.

We fix the cell tissue fixation or cell fixation means so that protein should not move after I start my work. So normally we use formally not para formaldehyde glutaraldehyde that can make a network between the protein and the cross-linked the proteins so that protein cannot move it stain its own position. So nuclear protein will stay in the nucleus they do not go up in a now Santo plasmic protein will be in the cytoplasm membrane protein will be in the membrane.

So first step is fixation second step is permeabilization what we do is and we make it permeable we do it permeable. And these permeabilization what you do is we make small very small pore here. How we treat the cell with very dilute and non-ionic detergent. Normally we use triton x-100, saponin which is very mild detergent and non-ionic, SDS is negatively charged it is also a detergent right.

But there are pure detergent which is non ionic neither positive nor negative but they are detergent they can dissolve the lipid bilayer of the membrane. And we use very diluted concentration and very short period of time it you have to do the experiment if you want to do it you have to standardize that but it is not long time. So that whole membrane should not dissolve. So, very brief exposure will make holes now again we are coming to same antigen-antibody thing.

Now if I add antibody suppose this is inner tube assume that only one cell is there but there are many thousands of cells and in here this is in a solution. And in this solution if you add antibody that antibody is specific to a particular protein that you are interested what will happen that antibody will go inside and that antibody will go inside but if you do not make poor antibodies very big molecule they cannot cross the membrane barrier.

So they cannot enter so if you make holes then it they will go and after going there if you incubate for hours or two hours or three hours what will happen they will bind to after they will enter and after that they will bind to wherever the protein is there it will bind but antibodies colorless you cannot see. So you have to find some device. So that you can see two possibilities are there one you can use same enzyme-linked antibody so you give some substrate.

So that color will develop whenever you will see the color like western blot whenever you see the color you can say protein is there. But here you cannot see if the color of a cell like this in naked eye, what you have to do if you want to see the cell you have to take make you have to take a slide you have to put some of the cell sample here put a cover slip on top of it and see under a microscope.

In microscope what you will see is you will see the cell and some part of the cell is colored and that color depends on what kind of substrate you have added where the color will generate whenever antibody is there color will be there that way you can see that protein is in the cytoplasm or in the nucleus but more sensitive and more sensitive and more accurate and better picture if you want to get what you have to do is?

What you need is the antibody which is labeled with fluorescence the flora probe you need a substance which will fluoresce and you know what is fluorescence right. You have to give some energy in the form of light so one light will fall on it suppose this is the process through the probe you one light will fall on it and that what we will do the outermost electron who excite to upper-level and then if while and that cannot I mean from its own state to a par state but it cannot stay there forever.

So it will come down to its original state while coming down it will emit some energy that energy you can see in the form of light that is fluorescence. So what will happen if you see so normally in every fluorescence what is there sorry every fluorescence what is there that there will be a excitation Maxima there were excitation Maxima that means you need a particular wavelength of light which will excite that fluoroprobes.

And then once it is excited it will come down and emit some light this is called emission Maxima so that means if every substance are all the materials that we have you know substance are not probable there are certain fluoroprobes. So depending on the emitted light the fluoroprobes may be green may be red may be yellow maybe orange. So different color you can get you can have blue so what kind of light if excitation Maxima will also change depending on what reagent you are using.

And the emission maximum is also change depending on what wavelength of light is emitted. We will see different color and that is in the visible range most of the time that is in the visible range. So now if I just delete this fluorescence mechanism part and then now if these antibodies suppose this is tagged with the fluorescence which is red what will happen? It will go and bind instead of enzyme everywhere it will be red in color.

So all the antibodies actually red in color again you cannot see this in naked eye or in the tube so what we are doing you are taking a cell sample you are fixing it for analyzing it then you are adding an antibody that you have because you already characterize your antibody is binding with antigen by Eliza and western blot. Now you want to see where the located then you are giving the primary antibody. Then you are giving the primary antibody.

This primary antibody with fluorescence that will go and bind same way that we see how you can see the cell will happen we need a microscope. But in this case what we need is we need a fluorescence microscope what we can see we if you see and turn on the light frozen slide so that means you have different color. So in fluorescence microscope you can excite for red you can excite for green you can excite for blue it all depends how much money you are spending.

If I spend more money more color you will get less money less color. Generally green, red and blue is normally there at least green and red is there. So to color detection fluorescence microscope it is the cheapest one. Much it is more expensive than the regular microscope visible microscope only but you can do. So what is there so you take a picture of the cell you take the picture of the fluorescence you wear you'll see only green or red or both.

And then both means two different I am coming and then you superimpose what actually you will see.

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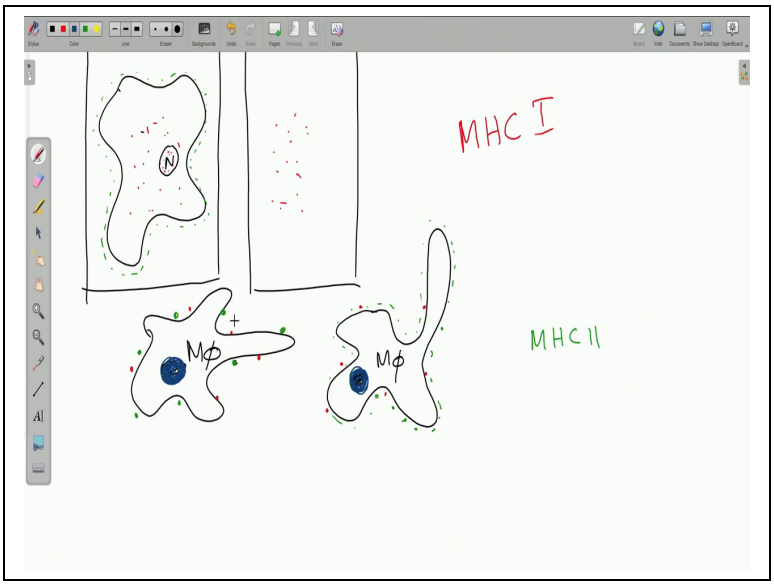

So this is what actually will see is so this is the cell so this is suppose a cell you are seeing under the microscope in visible light this is nucleus. In fluorescence you would not see the cell because it is in the black background you will just see some color some red dots. Now you take this picture because this is also in a frame right. So in this is in a frame this is another frame. So now if you superimpose that what I cannot do here if you superimpose that ultimately what you will see visible light all the dots are sorry all the dots are here.

If the dots are inside the cell you can say the protein is inside the cell. If the dots are inside the nucleus you can say it is in the nucleus. If it is in the membrane I am taking the membrane as say green color just to say we are in the membrane. Then you will see the nice outline of green bright fluorescent light in the membrane because you do not know where the protein is. Now imagine I am checking the presence of MHC what I was talking if you understand this part.

What will happen initially suppose to macrophage at two different stages this is the macrophage and this is another macrophages this is another macrophage, this is another macrophage I hope you understand what I said in the immunostaining so this is a nucleus. So the macro bond in the very beginning when it was just growing no battery is nothing you stain with a green fluorescence antibody against MHC.

So MHC is your target MHC 2 is your target and T-MHC 2 antibody which is you can by human or Mouse is pretty much available which is green in color you follow this technique what you will see initially we will see very few green, it is not absent and in your MHC class I showed you did not forget it happened few weeks back that MHC normally they express in dendritic cells and but they degraded by ebiqueti coordination by marchon protein I am reminding you again Marchon but when infection happen then this marchon disappear.

So there is no more negative regulation of MHC and more MHC right. So that if you want to see that real I mean whatever written in the book is right so you do the immunostaining and you will see suppose here there are only seven dots. But after incubating with bacteria if you repeat the same thing use anti MHC to antibody you will see many almost is increasing within a day it is nicely decorate the whole thing.

So if you see more position of more green or much brighter or the more intensity that means protein amount increase if there is one protein one and antibody one florescence. One protein one antibody which is attached to the fluorescence that means 1 molecule or 2 molecule of fluorescence right if you have 10 proteins 10 molecule of fluorescence. So, automatically 10 fluorescence molecule will give more light.

So if just by seeing you can tell which one is brighter which one is less bright. Now there are many software's are available so you can take the photograph or no you do not have to even take the photograph in the soft file or the digital image you can estimate what is the intensity or the pixel. So exactly you can compare between two fluorescence dot which one is brighter and how much brighter and you can tell that, that means the expression of that protein increase that much.

You can measure the whole cell you can measure a particular spot it all depends on what kind of microscope you have it all depends how good software you have. So this is immuno fluorescence, clear. So after this I will explain another technique which is very, very important all are important I am not saying the previously whatever I said is not important but important in what sense is very popular and used in many, many regular pathological detection and routine experiment.

As well as research because here what we can see is I can see 1, 2, 50 cells. If I would like to do something analyze something I can do it if I study this macrophage how many macrophage you can take picture 10 20 100 if you have time maybe 500 but if I say I would like to know the say 20,000 macrophage or 1 million macrophage what is their result. So sitting inside the; in front of the microscope and taking picture estimating them it is not. So individual cell or exact detail or much more detail you can understand by this.

But if you want to do the population study you need different technique clear. Before going to that I will just like to tell another thing suppose you localize the MHC 2 here with green. Now I would like to see MHC 1 also in that case so MHC 2 green color color now I would like to see MHC 1 and I will make it with red. So MHC 1 I will make red if I do the same use the same cell and use anti MHC 1 antibody levelled with red pure cells what I will see in this case the number is not going to increase.

Because bacterial update will not increase the MHC 1 right so what we will see MHC 1 if it is there it is not in many but maybe 4 or 5 just estimation it is just all they do not see that this is the real case this is just a cartoon to explaining. Here also you will see some MHC 1 here which is not going to change number of red dot here number of red dot here are more all the same because you are not checking same cell right.

This is one cell this is another cell but average number 5 to 6 or ten whereas green increased from 10 to 100. Two thing you can tell that MHC 1 is not increasing but MHC 2 is increasing that is one information and I do not know whether you realize or not at the same time two different protein you are localized. Two both the protein cell membrane protein but some protein some nuclear protein also you can localize suppose the red you are you have something another antibody which is blue and you can you can localize some protein in the nucleus which is appear as blue which is the Epirus blue.

So what will exactly see you have to take more pictures in this case you have to take four picture one visible cell suppose this is that dendritic or macrophage then one image of green

fluorescence another image of red fluorescence we should not change the field of the microscope the platform should fix just we are taking different filter different image visible red green blue four image then in software will superimpose them we can superimpose either one with visible we can superimpose green.

We can superimpose red we can superimpose yellow right but we can superimpose all of them and generate a composite picture where everything is set. So in one picture you can have two different proteins 3 different proteins all depends on how many antibody you have how many color you have how many times you can but this sounds very simple and straightforward but there is no science behind it because one antibody is going to buy an antigen everybody knows.

And if you tagged you with fluorescence it is going to give you a color known but doing this at cellular level so that each point is distinct cell is intact you need some arts in it you have to develop that art to do this nicely. Everybody can do that but to get a good picture good image nice what you want that to get that picture because you cannot control the cell right they will be have their own way so I could develop that art.

So I hope you understand this part now I am going to after this I am going to discuss about flow cytometry you must have heard about that flow cytometry and very commonly known as facts very commonly known as FACS fluorescence activated cell sorter but actually the technique is fluoro cytometry, clear. So actually the technique is flow cytometry.

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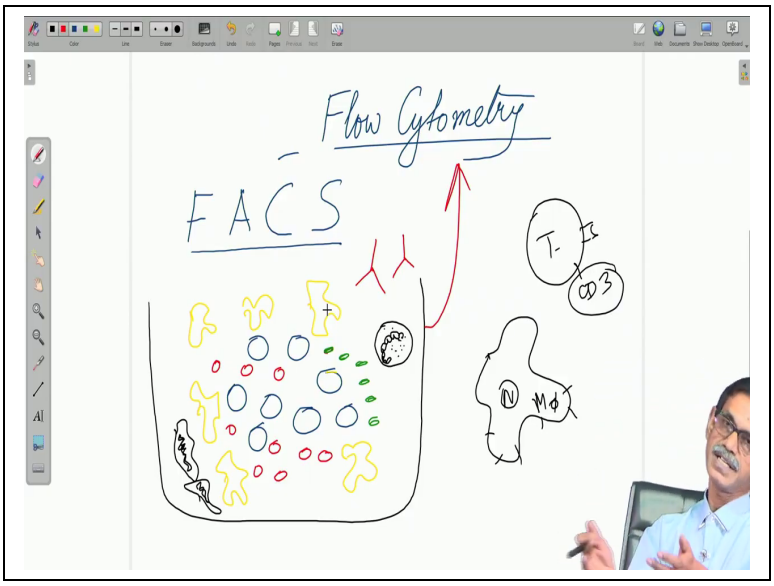

So this is Flow Cytometry another term is also called fluorescence activated cell sorter first before I proceed to this technique you have to remember that this is the technique flow cytometry is a technique. It is just a minute, so it is a technique but FACS is fluorescence activated cell sorter this is the name of the machine actual name of the machine or generic name of the machine is flow cytometer FACS is given by a particular company long time back.

But it is so popular now everybody is saying that I have to do fax that is not right so what this is doing this is the machine or the instrument by which we are going to do the population study that was just we are discussing few minutes back that immunostaining or immuno localization you know history all are same so what we are going to do is we will take a population. So we will take a population say lots of blue cells lots of blue cells and different kind of red cells and some green cells some green cells some yellow.

So I mean I I do not know whether you are noticing this are not my drawing I am not a very good artist but still you can see that not only the color is different their shape and size is also different some are bigger big and spherical some are small spherical some little elongated some is irregular shape. While we can get this kind of cell where we see this kind of cell this kind of cell we see in blood we have a pretty small platelets we have RBC small and then we have lymphocytes we have macrophage, dendritic cells which are this kind of thing.

Another kind of cell is there so which I can tell you like say not many colors say black. So they are elongated big cell and not only that they are nucleus is very complicated. So this is the cell and you see there globular nucleus where it will say polymorphonuclear cell that is also present in black that inside is very much complicated. Lymphocytes inside is not very much complicated because there have nucleus and mostly very straightforward like most of the cells.

But polymorphonuclear cells they are they are very complicated inside. So this type of cell mixture of cells so when I am saying last staining all our macrophage all look same identical. But when you have so many variety of cell if you just consider the blood cell is a very good example of that RBC, platelets, macrophage, monocyte, lymphocyte, b-cell, t-cell, NK cells and then you have the basophile, polymorphonuclear cells neutrophil right.

So they are big they are complicated from inside and they are this different shape and size also not only they are circular some are very regular shape. So FACS is the machine or the flow cytometry is the machine where by using them you can identify which one is what, not exactly seeing it machine you will do that you have to understand or you have to know how to read the outcome of the machine.

Then you can tell which one is one and now not only the size and shape difference I also give different colors here while drawing this because is it possible now by you me no standing that what we learn few minutes back. Like I can make some cell blue I can make some cell red I can make some cellular some cell orange some cell green, so along with this variety of size and shape and complexity if I allow or if I use the immunostaining or immuno coloring histochemistry method.

This population I can make variety of cells like if I use anti t-cell receptor antibody it will stain only in t-cell. If I use anti b-cell receptor antibody it will stain only b-cell same way there are some marker for macrophage. So I will use that some for dendritic cells is that so if I have a definite and genuine antibody for any particular type of cell I can use that to label that cell only earlier I just discussed.

So you say anti macrophage antibody which is say this is macrophage. So this is a macrophage and this is nucleus this is a macrophage. So macrophage I know there a specific protein which present only in macrophages same way if it is t-cell, T t-cell receptor present only in t-cell what else present in t-cell only there are another receptor you know CD3. So CD3 is T cell specific if I have anti CD3 antibody.

Suppose anti CD3 antibody which is labeled with red color clear. So if I mix in blood what will happen if I give this red antibody I am not showing the fluorescence if I just say antibody is red if I give the red antibody which is anti CD3 and mix in blood what will happen this antibody will go and bind only the t-cell suppose the blue or the t-cell she will go only and make the blue to red process and if I see under microscope I can tell you but how many cells are colored?

This machine or the flow cytometry will tell you exactly what percentage of cell is this rate and if can tell you say 20% of the cell is or 30% of the cell is red that means 30% of the total cell is t-cell so that you can figure it out and also you can figure it out there is according to separate them according to their size and say you can estimate the number of cells. You can estimate many other things you can do many things with the flow cytometry.

But I mean we are going to restrict on illness. So today this is just the introduction of flow cytometry in next lecture I am going to talk about mechanism and how it work still then, see you.