Experimental Biotechnology
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Lecture - 34
Microscopy (Part 1)

Hello everybody, this is Dr. Vishal Trivedi from Department of bioscience and bioengineering from IIT Guwahati. And so, far what we have discussed we have discussed about the culturing of the mammalian cells. And in the previous lecture, we have also discussed about how you can be able to fractionate the mammalian cells as well as the prokaryotic cell with the help of the different types of centrifuges.

Either you use the differential certifications or the density gradient certifications, no apart from usage of the centrifuges or to separate the cells into different fractions, we can also localized or we can also locate a particular cell within the particular protein within the cell with the help of the microscopy. So, the cells what you are culturing with the help of the different types of media can be visualized with the help of the different types of microscopes.

So, in today's lecture, we are going to discuss about the microscopes and how you can be able to use these microscopes to localize a particular protein within the cell or how you can be able to utilize these microscopes to perform different types of cell based experiments.

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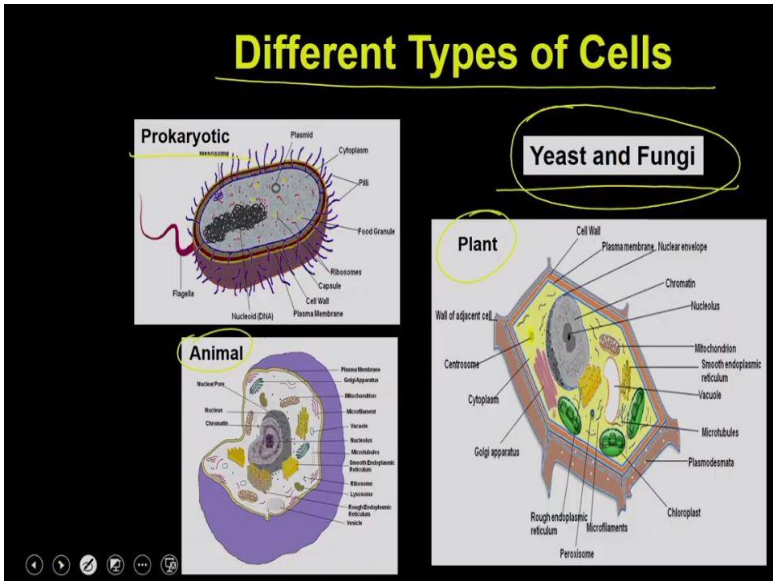

So, I think we have discussed about the different types of cells, we had discussed about the prokaryotic cells as well as the eukaryotic cells within the eukaryotic cells, we discuss about the yeast animal cells as well as the plant cells and to visualize these cells we can use the different types of microscopic tools. So, the microscope is a instrument which actually can be used to visualize the cells which are very, very small and there are different types of microscopes. So, let us go through with those.

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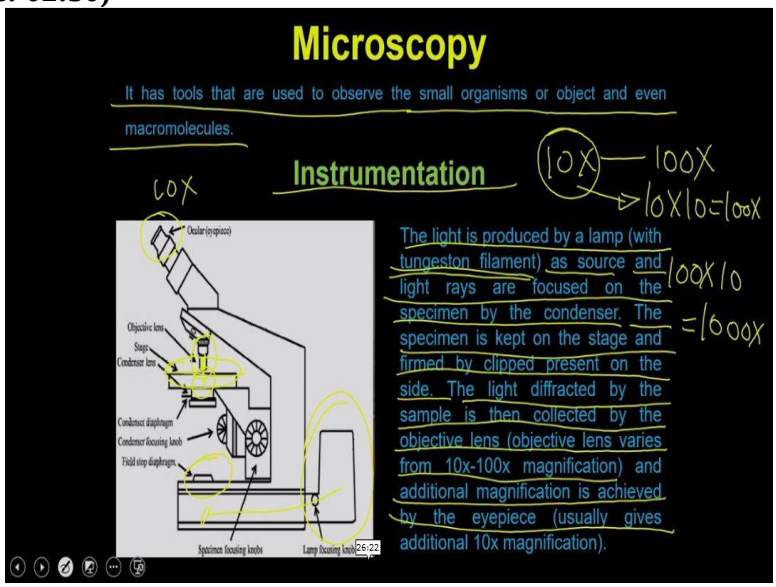

So, microscope is a tool that is used to observe the small organism or object even the macromolecules in a typical microscope, what you have is you have a light producing source for example, in this case you have a light source. So, in this place you have a bulb, which is actually been made up off of tungeston. So, in a simple microscope, which is actually this is a light

microscope, the light is produced by a lamp with the help of the tungeston lamp as a source and the light rays are focused on to the specimen by the condenser.

So, the light comes from here and then it is actually been focused with the help of the condenser and the specimen is kept on to the stage and formed by the clips present on to the side. So, here you can be able to keep the specimen and then you can keep the specimen forms with the help of the light the clips, the light diffracted by the sample is then collected by the objective lens objective lens are actually in the range of 10 to 100x which means, once the light is being reflected by the objects it is actually going to be collected by the objective lens.

These objective lens could be from 10x to 100x, which means, it is actually going to magnify the object by a factor of 10. But there is an additional magnification is achieved by the eyepiece which is usually by the 10x. So, in case you are looking at a sample with the objective of 10x it is actually going to give you a magnification of 100x. Similarly, if you are looking at the object with 100x so it is actually going to give you the total magnification which is 1000x.

So, this is a simple microscope a light microscope where you have a light source, this light source is going to be focused by the condenser and then this condenser actually illuminates the objects which is been or the specimen sample which is kept on the stage. And once the light is diffracted from the sample it is been collected by the objective lens and these objective lens can be off 10 to 100x and then the total magnification what you are going to achieve is by the magnification by the objective lens as well as the magnification by the eyepiece. Mostly the eyepiece are 10x so, you are going to get a magnification of the 10 into whatever the magnification of the objective lens.

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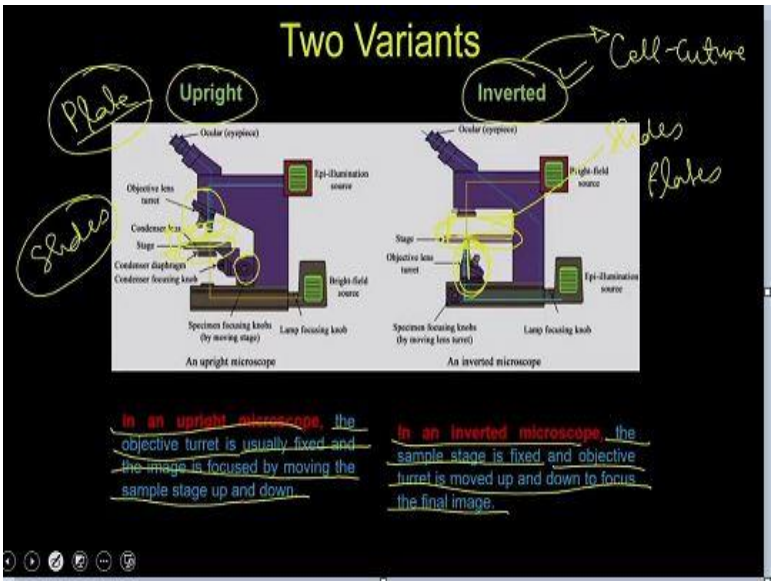

This instrumentation could be of 2 different types either you can have the upright microscope or you can have the inverted microscope. In the upright microscope the overall scheme remains the same except that the objective the turret where you have the objectives are being fixed is usually fixed and the image is been focused by the moving the sample stage up and down, which means in a upright microscope, the objectives are remained fixed.

Whereas the sample you are going to keep on to the stage and the stage is going to go up and down with the help of the adjusting knobs. So, you have a knob here which actually can be rotate into the clockwise or anti clockwise and with the help of that, this stage can go into the up and down directions and that is how you are actually going to focus the samples, which means the field of depth is going to be less in the case of upright microscope and you are not going to have enough space to keep a big size objective.

For example, in the upright microscope you will not be having a flexibility to keep the plates in most of the upright microscopes, you are actually going to keep only the slides only so that you can be able to visualize the slides because the space between the fixed objective and the moving stage is going to be very narrow. So, you cannot be able to keep the plates in comparison to that you have an inverted microscope, the inverted microscope the overall light path and everything remains the same except that in a inverted microscope the sample stage is fixed.

So, you are going to keep the sample on a fixed stage and you are going to have the moving objective. So, you can imagine that the whatever the design you have, it is exactly the opposite in the case of inverted microscope, and the objective turret is moving up and down to focus the final stage because the objective is moving and you have the fixed stage there is no problem of keeping any object including the slides or the plates onto the top of the stage because the distance between this and this remains the fixed.

But on the top of the stage you have the enough space to keep the objective of any thickness which means you can keep the slides you can keep the plates you can keep anything and that is why the inverted microscope is actually useful for observing the cells under in within the cell culture labs, which means you can actually use the inverted microscope to observe the cells file they are culturing into that culture dishes or you can actually observe the cells.

Suppose you stain the cells with some fluorogenic substance or some fluorogenic dye then you can be able to directly you know observe those cells under the microscope in the inverted microscope whereas in the case of upright microscope, you might have to take out those cells, then you mount it onto the slide and then you can be able to observe because the distance between the objective and the stage is very narrow. So, this is the comparison of an upright microscope versus inverted microscope both the microscope have its own advantage as well as the disadvantages.

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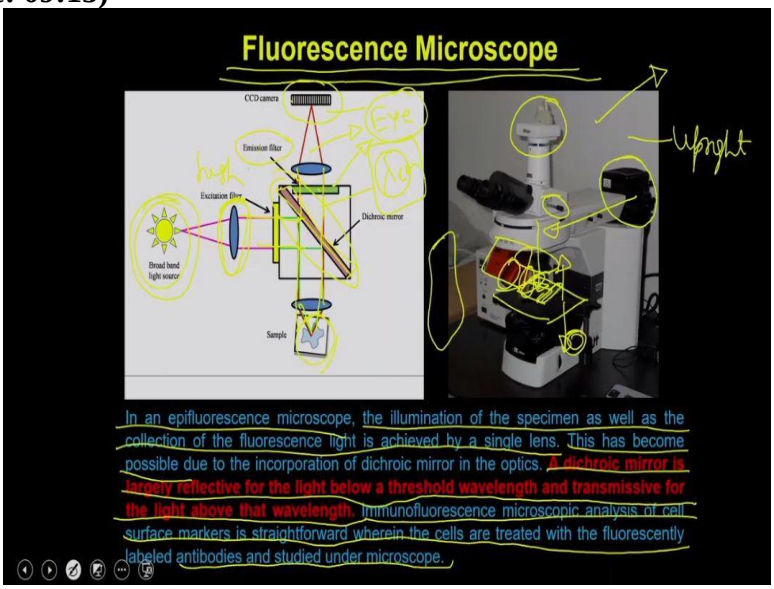

Apart from that, you also have the fluorescence microscopes. So as per the light source, you can have the light microscope or the fluorescence microscope in a fluorescence microscope or in an epifluorescence microscope, the elimination of the specimen as well as the collection of a fluorogenic light is achieved by the single lens. So you can see that this is a typical fluorescence microscope. So this is a upright fluorescence microscope.

Where you have the stage which actually can so this is the stage which actually is not fixed which actually can go up and down with the help of the adjusting knobs which are present here. And these are actually the turret on which the object is are being placed. So you can keep a slide or you can keep the specimen here and then you have the clips with the help of the clips, you can be able to fix your sample here. And then what you see is that if you have a light source and through light source.

From this light source, the light comes from here, and then it actually eliminates the samples. And because the user is going to be on this site, that is why if there will be any diffracted light coming from the sample, to protect the user, they also have a shield so that it should not directly goes into the to the user. So in a typical light scheme, what you have is that you have a light source, which actually going to give you light of the broader wavelength, and then you are going to have the excitation filter.

So as you know that fluorescence in a fluorescence phenomena what you have to do is you have to excite the samples with a excitation wavelength, and then the sample is going to emit a wavelength which is going to emit a light, which is going to be of a higher wavelength. And then that wavelength has to be collected with a emission filter, and then that you can be able to visualize with the help of the different types of objectives as well as the eyepiece.

So what will happen is that you have a light source, which actually going to give you a light of the broader wavelength, then you can actually focus that with the help of the excitation filter. And then from the excitation filter, the light will go since the light is go, and then it is going to hit by a dichroic mirror, because the light is going to be of a high wavelength, it actually going to reflect and going to eliminate sample now from the sample, the light is going to be produced.

So this light, what you see is a red color light is actually going to have the lambda emission. So lambda emission is going to be off a higher wavelength. So once the higher wavelength goes, it is actually again going to be hit by the dichroic mirror. And by this time, instead of going to be reflected, it actually goes straight. And then you are actually going to have the emission filter. And that emission filter is again going to filter, whatever the wavelengths are coming from the sample, and then it is actually going to show you the light of your desired wavelength.

And that is how it actually will go to the user to observer. So this is actually go to the camera which is actually been placed here. But if you use this knob and you change the field of view, it actually can go to the eyepiece, and that is how you can be able to observe these fluorescence slide. So, this diversion of the light with the help of a dichroic mirror is only possible because the dichroic mirror is largely reflective for the light below a threshold wavelength and a transmissive for a light above that particular wavelength for dichroic mirror is a special mirror.

Which actually reflects a light when the wavelength is of a lower sight. So it actually reflects that light, and then that is how the reflected light goes and hit to the sample, but when a larger wavelength comes out from the sample, then for that particular wavelength, the dichroic mirror is transmissive. So, it actually allowed the transmission of that particular light and that so, it is actually been focused again by the emission filter or it is been filtered by a emission filter.

On that is how it goes either into the eyepiece or to the CCD camera for collecting the image. The immunofluorescence microscopes analysis of the cell surface marker is a straightforward whereas the cells are treated with the fluorescently labeled antibodies and studied under the microscopes. So, because you can be able to illuminate the sample with a standard fixed wavelength and you can be able to acquire the light from the sample with the help of these fixed wavelength, you can be able to utilize the fluorescent microscope.

If you tag the cells with a fluorescently labeled antibodies. So, that you can do simply by the staining the cells with it fluorescently labeled antibodies or to the fluorescent label probes.

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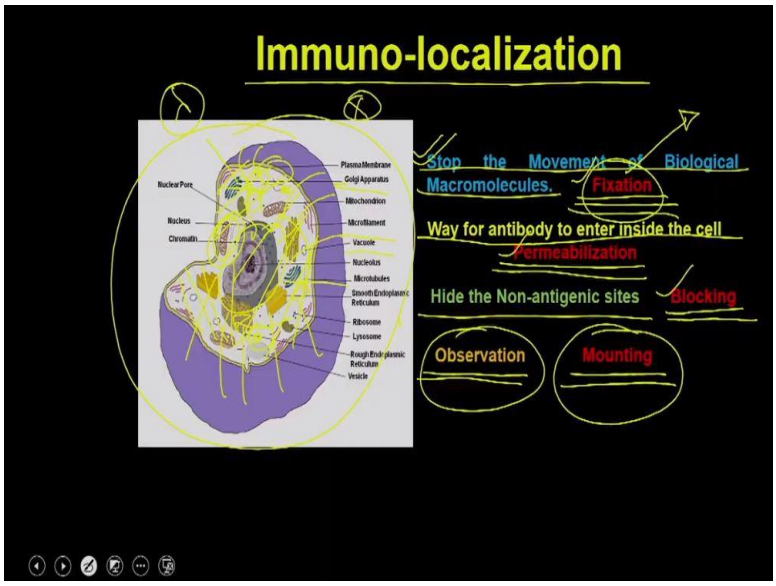

So, you can imagine that you have mammalian cells and just give me an example of a mammalian cell how you can be able to perform Immuno-localization studies, but that can be replicated or that can be done with few modifications even for the plant cell as well as the (())(14:43) cells or to the bacterial cells. So in a plant cell, what you have is you have the different types of organelles like the nucleus, you have the mitochondria, you have you know the plasma membrane then you have the Golgi bodies and all that kind of thing.

So, and all these molecules are actually under the dynamic equilibrium, which means there is no fixed place for a mitochondria to remain there or there is no fixed place for the other kind of organelles, because all these organelles are suspended within the cytosol and they are keep moving within the cell. So, to if you would like to do a immuno-localizations the first thing, what you have to do is you have to stop the movement of the self.

And that is going to be the first step, if you are interested to the immune-localization, this means the first event what you have to do is, you have to stop the movement of the biological macromolecules. And not only the organelles, the macromolecules are also been freely moving, for example, within the mitochondria, you have the electron transport chains, but the other molecules like the enzymes of the cycles and all that is actually freely moving within the mitochondria. So, if you are interested to localize that particular enzyme.

It can actually move you know, change its location or it actually can change its position. So, for example, if I am localizing an enzyme, and it is localized here at time x, after 10 minutes, it could be localized somewhere here actually. So, that is actually is very, very problematic, because if you are reporting that, the particular molecule is localized to the plasma membrane, and then after 10 minutes, it can actually because it is under the dynamic equilibrium with the cytosol, the molecule can go to the next locations.

So, that is why it is important that first you stop the movement of the organelles, as well as the movement of the macro molecule what is present inside the cell. So, that you are actually going to done or going to do with the help of procedure or the protocol called fixation. Once you have fixed the cells, then the cells are actually going to cease its biological activity, it is actually going to die and then all these organelles are actually going to be cross linked by the different types of fibers and that is all the molecules are going to be remain as like a fixed situation.

Which means they are not going to be allowed to move so that you will be able to see the localization under the fixed situations, there are conditions were actually you can do the localization even under the lifecell conditions, but those are different conditions and there are the different way in which you can be able to do that, since you are done that then you have to make the way because you know the cells are not permeable for the antibodies, which means the antibodies are cannot enter into the cells.

Because the mammalian cells or the plant cells or even the bacterial cells are only permeable for the hydrophobic molecules as well as the small molecules, but they are not permeable for the larger molecules like antibodies to make the part for the antibodies that is the second step what you have to do. So, in the second step, you have to make the way for the antibody to enter the cell and that is being done with the help of a step called permeabilization.

Now, once you have permealized the cells, you are actually going to make the tracks within the cell which means using these tracks or using these paths, use the antibodies can enter but once the antibody is going to enter the cell, it is not going to interact only with the antigen of your interest is also going to interact with all the proteins what is present in the cell. So, to avoid that,

you also have to hide the non-antigenic sites and that you have to do with the help of the blocking. So, once you have done with the fixation permeabilization and blocking.

Then you are actually been ready to stain the mammalian cells with the primary as well as the secondary antibodies. And then after that, you have to do the observations means you have to see the cells. So, for observing the cells, you have to again do a procedure which is called as the mounting which means you have to mount the cells onto a cover slips and then you can keep the cover slips under the microscope and then you can be able to visualize there is a possibility that you can actually use the inverted microscope and then you do not need to do a mounting.

But in that case also you might have to keep some solutions. So that the sample should not get destroyed when it is getting illuminated with a high beam of the fluorescent light or high beam of the light which is coming from the source. So let us discuss all these steps in a more detailed.

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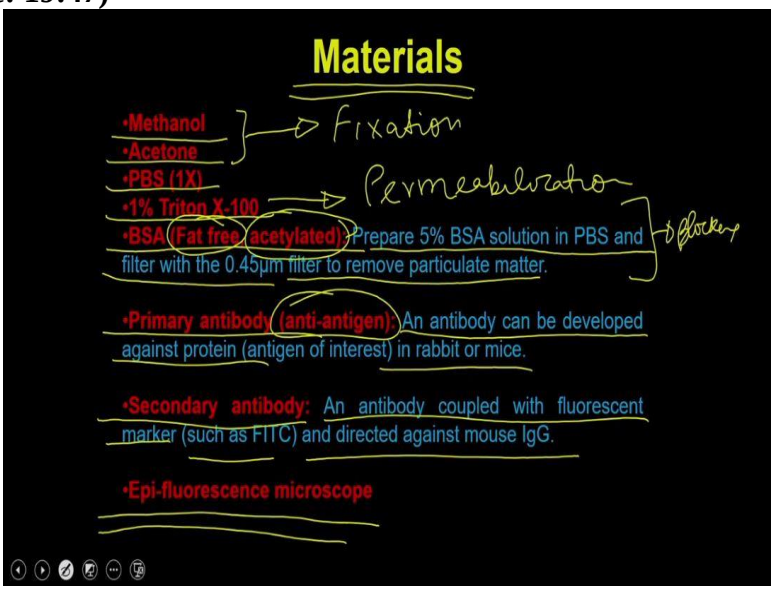

So the material what is required for you if you want to do a immuno-localization studies you require the methanol and acetone so that you require for during the fixation then you require the phosphate buffers saline triton x100. So triton x100 is required for the next step which is called as permeabilization then you require the BSA which is actually the fact free and the acetylated BSA, because the acetylated BSA is better in terms of doing the blocking steps.

So, you have to prepare the 5% BSA solution in PBS and filter it with the 0.45 micron filter to remove the particulate matter and BSA is required for doing the blocking then you require the primary antibody the antibody, which is you are going to use for against the antigens, the antibody can be developed against the protein in the rabbit or the mice, then you require the secondary antibody a secondary antibody coupled with the fluorescent markers.

Such as the FITC and directed against the mouse IgG, or the rabbit IgG depending on in which you have actually generated the antibodies, and then you require a microscope which is a Epi-fluorescence microscope. Let us see how you do all these procedures. So, in terms of the procedures, you are actually going to do this step.

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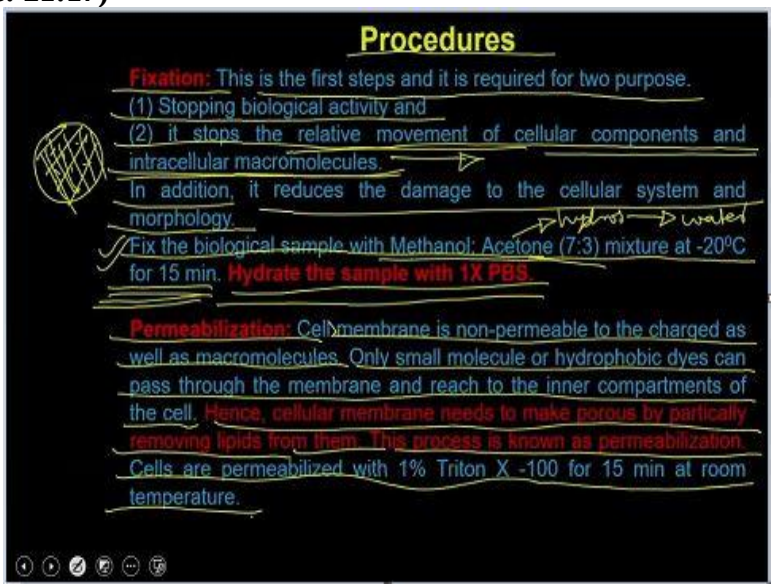

The first step is the fixation. And this is the first step it is required for the 2 purposes, in the first step, you actually have to stop the biological activity, because, why you are supposed to stop the biological activity, because if you are doing the procedures, during this procedure, actually the cells are going to be remained without nutrition. So, if you keep the cells without nutrition, the cell is going to be in a field of stress and if you in under any stress, the cell is going to start producing the very large quantity of free radicals.

And because of that, the cell is going to be lysed in due course, for example, if you are staining the cells and you are doing all this procedure, without doing the fixation and without stopping the biological activity, what will happen that you know, the free radicals are going to be

produced because this procedure are normally takes approximately 12 to 24 hours. So in during this procedure, if you are keeping the cells biologically active, and they are under the starvation conditions, then they are actually going to be lysed.

And then you are actually going to not get the so they will not going to maintain the cellular integrity and that is at the end, what you are going to get is you are going to see scrambled cells and you are not going to be able to localize and that is why you have to stop the biological activity, and then you also have to stop the relative movement of cellular component as well as the intercellular macromolecules, because that is very important that you stop the movement of the organelles, so that they will remain fixed at particular locations.

And then within the organelle also, you have to stop the movement of molecules. So that you will be able to precisely say that were that particular macromolecule was present when you are doing the localization studies, which means suppose you have treated a cells with x compound, and then you are trying to do a localization. But if you do not do the fixation step, the localization of that particular protein or that particular factor is going to be changed, because they are under the dynamic equilibrium.

So the protein can move from the membrane to cytosol or cytosol to membrane and all that kind of thing. So that actually is going to create a lot of artifacts while you are doing the localization. And that is also not going to help you in terms of the understanding the biological phenomena. For example, if I am treating a muscle cell with the insulin, and I am looking for what will happen or what is the localization of the hexokinase, then in that case, I have done the treatment only for 5 minutes. So after 5 minutes, I should stop the biological activity.

So that as well as I should fix the cells, so that I should be able to very precisely say that the hexokinase was present in cytosol, it was not present anywhere else. Because if I do not do the fixation, and I will keep doing the procedures, then the hexokinase may actually get degraded, it may actually can move from one place to another place, and it can move from one compartment to another compartment and so on. So that is why it is important that you stop the relative movement of the cellular components as well as the intercellular molecules.

In addition, it reduces the damage to the cellular system as well as the morphology. So the third point is that because you are going to fix yourself, you are actually going to stop the cellular disintegration because as I said, you know, when you are creating a stress inside the cell, eventually the cell is going to get lysed and that is how it is actually going to lose its morphology. So that are the 3 important reasons why you have to do a fixation.

You fix the samples with the help of the methanol acetone mixture at minus 20 degrees Celsius for 15 minutes and when you do the fixation what happened is that suppose this is a cell actually and you have the different types of organelles what is present. So, you know that the cell is filled with the protein molecules. So, these protein molecules are actually containing the amino acids and these amino acids are containing the side chains and these side chains are actually contained for example, the lysine and arginine are actually containing the amino groups.

And these amino groups are being cross linked with the help of the methanol as well as the acetone mixture. So, when you are treating the cells with the methanol and acetone, you are actually you know, you are actually cross linking the all the protein what is present. So, if you cross link all the proteins, first of all the protein is going to lose its biological activity or the enzymatic activity on the other hand, because all the proteins are going to be cross linked.

So, you can imagine that you have made actually the fibers within the cell with and all these fibers are nothing but the cross linking of all the proteins. So, once you have a very big mesh of the proteins, what is present inside the cytosol as well as in other organelles, it is actually going to make it as an intact integrity. And then after that whatever you do, it is actually not going to lose its cellular integrity.

On the other hand, as soon as you have actually cross linked these enzymes, they are actually going to lose its biological activity or the enzymatic activity and the end the cell is going to lose its biological activity, because the cell is a hydrated material and because you are treating this material with the methanol and acetone which is very hygroscopic. So, they are actually going to withdraw a large quantity of water also from the cell.

So, that is why and the water is very important for you to see the localization because if you remove the water from the cell, you are actually going to compromise the cellular morphology because when you remove the water it is actually going to shrink and because of that, you have to hydrate the sample with the 1x PBS so that all the water is going to be come back and all the water is going to be replaced with the phosphate buffers.

So that the cell will actually going to acquire his normal morphology and in addition to that, it is actually going to be fixed then the next step is the permeabilization. So, cell's membrane is non permealized to the charge as well as the macromolecules only a small molecule or the hydrophobic dye can pass through the membrane and reach to the inner compartment of the cell. Hence, the cellular membrane needs to be make porous by practically removing lipid from them, this process is known as the permeabilization cells are permealized with the 1x triton extended for 15 minutes at room temperature.

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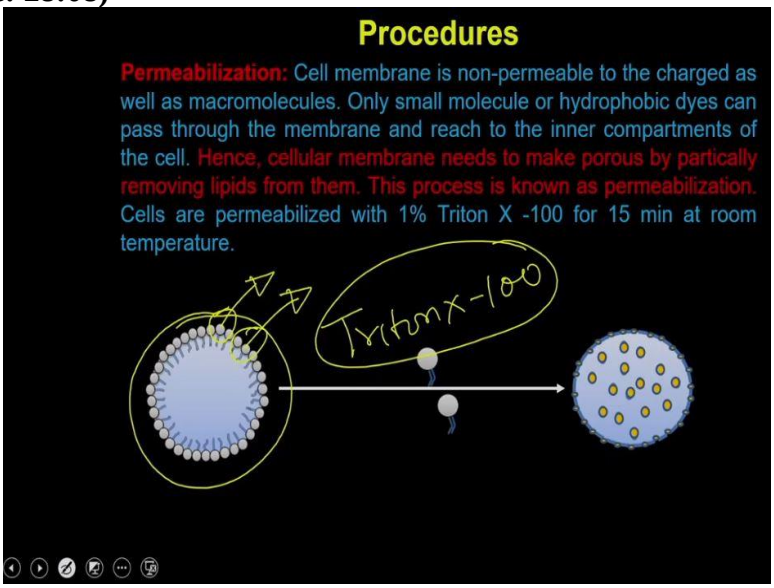

Let us see how the permeabilization works. So, you can imagine that this is actually a cell and this is a plasma membrane, which is actually containing the lipids. So, what happened is when you are adding the triton x100, so triton x100 is a detergent. So, what will happen is the detergent is actually going to start dissolving these, lipids and if you do are triton x100 in a controlled fashion, which means you are not going to do a lot of treatment for a very, very long time.

For example, in this case, we are doing a 1% triton x100 for 15 minutes and that can be optimized even for different cells, you are actually going to remove the lipids at a very discreet location. So, because of that, it is actually going to make the holes for example, once you do that, you are actually going to remove the lipids at a different positions. And that is how you are actually going to make the hole which are good enough for taking up the antibodies.

But they are not good enough so that they are going to lose the organelles as well as the other macromolecules structures which means these holes are good enough for the delivery of the antibodies, but these holes are not going to allow the cell to lose its organelles. On the other hand the cell is already fixed. So, even if you made the hole that will allow the antibodies to go inside by utilizing the channels what is being formed inside the cell, but the structures are already been cross linked. So they will not come out from the holes.

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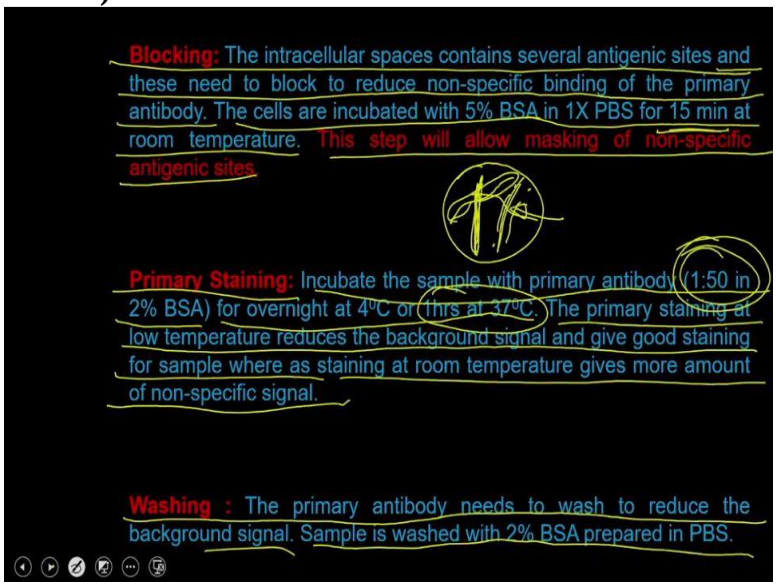

Now after that you have to do a blocking step. So intercellular spaces contains several antigenic sites. So you can see that in a cell you are actually going to have the channels which are being formed and utilizing these channels the antibodies are going to form going to travel to your antigen of interest, but these channels are actually having the proteins, different types of proteins. And the antibodies eventually are going to bind these proteins in a nonspecific manner, because the concentration of these proteins are going to be very, very high.

So, even if the protein even if the antibody is very specific, there is a chances that this antibody is going to interact with these nonspecific antigens. And that is how you are actually going to see a large background. So, to reduce the background, what you are going to do is you are going to block or you are going to coat these path where the antibody is going to travel with the help of a nonspecific proteins.

So that is why these antigenic sites are going to be blocked by reducing the nonspecific binding off the primary antibody, the cells are incubated with 5% BSA in PBS for 15 minutes at room temperature. Now, this step will allow the masking of the nonspecific antigenic sites. So that is how you are actually going to reduce the background signal. So, that is the step which actually can be optimized in such a way so that you will be able to see a specific signal for your antigen and you can be able to reduce a nonspecific signal.

So, this is not a fixed rule that you do it for 15 minutes, it can be optimized. In some cases people do not use the BSA, they use the some more complex protein solution for example, you can use the serum you can use the other protein sources as well, because the BSA is very simple protein. So, it may actually not be able to you know give you the very good blocking compared to that if you use the serum and other kind of complex biological samples.

And that is how you actually optimize, so that you get the specific staining for your antigen of interest, but at the same time you block the nonspecific signal to reduce the background. Then after that, you are going to do a primary staining so incubate the sample with the primary antibody mostly 1 is to 50 dilutions in a 2% PSA for overnight in 4 degree or you can do a 1 hour at 37 degrees Celsius, the primary staining at low temperature is reducing the background signal and gives the good staining for sample.

Whereas, the staining at room temperature gives the more amount of nonspecific signal. So, when you do the primary staining, the primary staining has to be done with the help of the primary antibody what you have developed in the rabbit or mouse or whatever you have actually purchased from the company and that you have to do as a consultation of 1 is to 50. So, this is

not fixed that you do a consultation of 1 is to 50 dilutions you can have to optimize this and depends on the antibody titer of that particular antigen.

Because if the antibody titer is very high, then you can be able to even afford to go up to 1 is to 200 or 1 is to 500 but if the antibody titer is very low, then you have to go with the 1 is to 100 1 is to 50. And the other point is that you have to do the immunostaining at a very low temperature so that you will be able to stop the nonstick interactions because at a low temperature, you are actually only allowing the antibody to interact with the specific antigens and you are actually you know, avoiding the nonspecific interaction.

Whereas, if you are quickly want to test the protocol, you quickly want to test whether the antibody is staining my antigen or not, then you can do the 1 hour 37 degrees Celsius that actually is going to tell you whether the antibody what you have developed is good enough to do the immuno-localization in the cell or not, but it is eventually going to give you a very high background. So, once you are sure that it is actually going to give you the good staining.

Then you can actually perform the same experiment under the in low temperature at 4 degrees Celsius. You can do then the washing the primary antibody needs to wash to reduce the background signal and the sample is washed with the 2% BSA prepared in PBS. So the washing step is also a step where you actually can do some amount of optimizations. So that you can be able to reduce the background signal while maintaining your interest of your signals.

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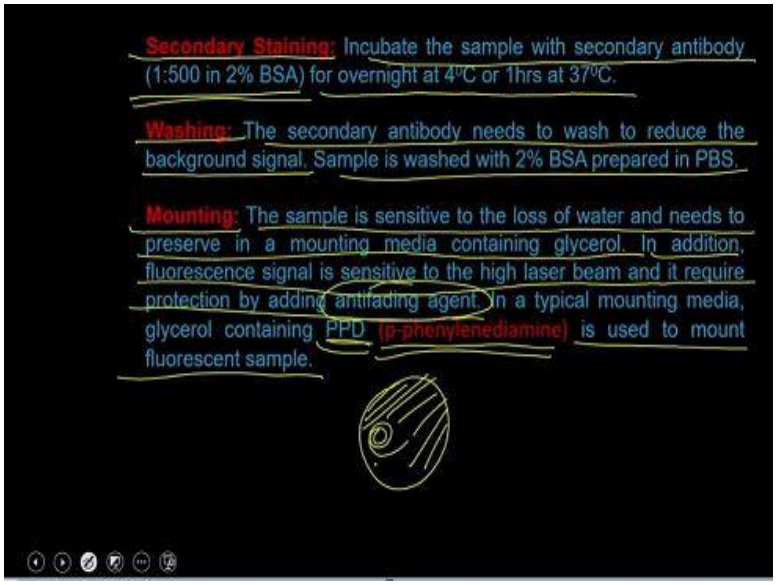

Now once you are done with the washing, then you can do a secondary staining. So incubate the sample with a secondary antibody mostly in a 1 is to 500 into a 1 is to 500 dilution in 2% PSA for overnight or at 1 hour for 37 degrees Celsius then you are going to do a washing. So the wash secondary step need to wash to reduce the background signal and the sample is washed with 2% BSA prepared in PBS, then the last step is the mounting the sample is sensitive to the loss of water and need to preserve in a mounting media containing glycerol.

In addition, the fluorescence signal is sensitive to the high energy laser beam and it require protection by adding anti fading agents. So, when you are doing a mounting since in this case we are doing the immunofluorescence and the fluorescent the light instance is very bright feet actually can quench your signal which means, it is actually going to you know illuminate the flour of 4 to such a high beam that it eventually instead of giving you the light it actually going to destroy it and this phenomena is called as the quenching.

Which means, if you are illuminating a particular biomolecules or if you are eliminating a probe with a very high energy beam, so, instead of giving you know the fluorescence, it is actually getting oxidized with the help of that high energy and that is how it is actually going to be destroyed. So, to avoid that you also have to mount the sample with the help of anti-fading agents. So, anti-fading agents are the sample or antifading agents are the compound which actually toned down the signal.

And it actually absorbs of whatever the extra heat what is present in that high energy beam. So, because of that, it actually allows the sample allows the probe to be get illuminated or the flour what you have added to be illuminated, but it does not allow the extra energy to be acquired by the floor for so that it will get quenched. So one of the classical antifading agent is called as the PPD or the para phenylenediamine and the para phenylenediamine is actually going to protect the sample from getting the quench.

So, you can actually mix the PPD in a mounting media and it is can be used to mount of fluorescent samples. So, you can imagine that if you have a sample in a fluorescent dye in a sample and you have a coverslip so, what will happen is the PPD is actually going to present throughout this sample and that is how it is actually going to absorb extra light what you are actually using to illuminate the sample and it is only allowing it is just like you know filter. So, it actually reduces the intensity and it reduces the extra energy, but it actually allowed the flow for to give you the fluorescence instead of getting quenched.

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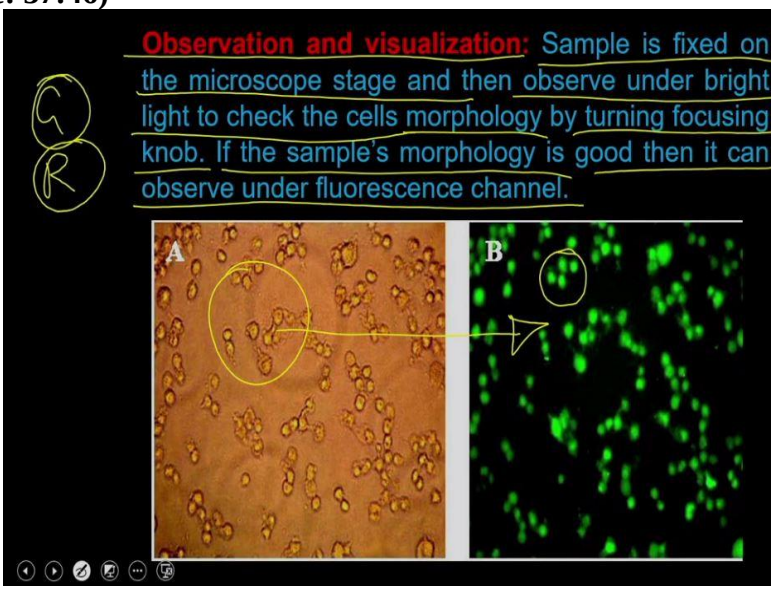

Now this is a typical observation and visualization. So sample is fixed on the microscope stage and then you observe under the bright light to check the cellular morphology by turning the focusing knob. So, this is the bright light image and if the sample is morphologic is good, then it can be observed under the fluorescence channels. So, what you can see is that I am observing the same slide under the fluorescence channel and so, all the cells are showing me a fluorescent signal and it is actually showing me a signal off the antigen.

If I go with the more higher magnifications and if I go with the more number of fluorescent for example, if I use suppose I am interested to see whether the protein of my interest is present in the mitochondria or lysosomes and Golgi bodies and all that, then what I can do is I can stain these cells with the particular organelles specific probe as well for example, I can use the mitotracker red, so in that and I can use the fluorescently dye like green.

So, what will happen is if I do so, and if I see a co localization of the green in red, then that actually is going to prove that the protein of my interest is present in the mitochondria, if I use the similarly, if I want to do other kind of experiment, I want to see whether the protein of my interest is present in the nucleus, then what I can do is I can just stain the cells with a nuclear dye for example, I can use the (())(39:14) or I can use the etbr or I can use the propidium iodide and or the accreting orange and that actually is going to stain the nucleus.

And if I see a co localization which means if I see a superposition of the 2 signals, then I will say that my protein of interest is present inside the nucleus. In fact, you can actually even fine tune you can actually go further down and say, whether my protein is interacting with the electron transport chain of you know the mitochondria or not. So, if I do a immunostaining the same procedure if I do immunostaining for the some of the complex is what is present in the electron transport chain. And if I do protein of my interest with some other enzyme.

So, what I can do is I can use one antibody with the green I can use the one antibody with red and if I see a signal overlap, which means wherever the green is present the same place you can see a red signal. So, that is a way it is actually going to say that I am showing the co localization of these 2 signals, which means my protein is actually been present very close to the electron transport chains, you if you remember that these are the fluorescent signals.

So, they have these, they are not going to say whether how far they are, but they are going to be in the within the range of 10 to 50x storms that is all it is actually going to say they are very close by if you want to be very pinpoint and you want to say well, how close they are, then you can actually be able to use very high resolution microscopy as well. In that case, you can actually do

a localization with the help of the electron transport chain with the help of the electron microscopy.

And that actually is going to tell you very precisely how far these 2 proteins are present within the electron transport chains that anyway we are going to discuss in a subsequent lectures. So, let us see how you can be able to utilize the fluorescence microscopy to understand the some of the biological phenomena.

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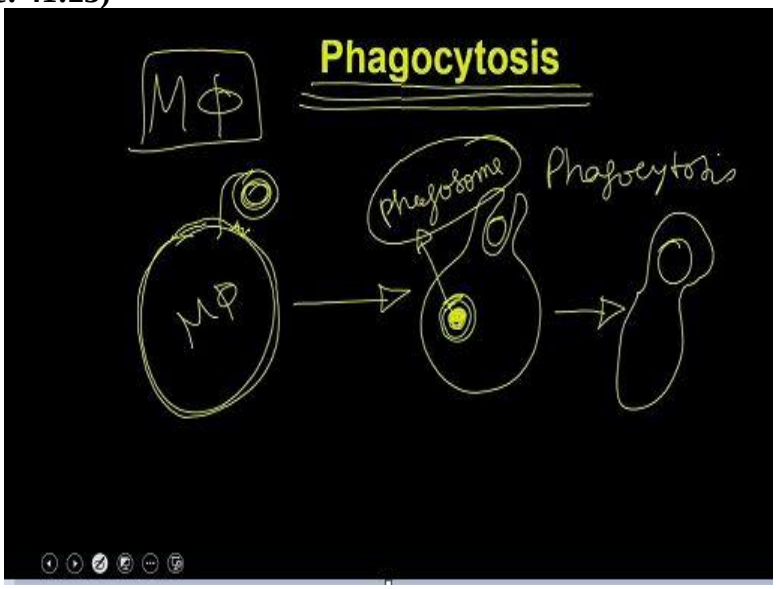

So, one of the basic biological phenomena is called as phagocytosis. So, phagocytosis is always been done by most of the immune cells in especially like macrophages. So, what happens is, when the macrophages are being found a bacteria So, suppose this is a bacteria, so, if the macrophages are found that this there is a bacteria and so, the phagocytosis means this eating by the cell, which means the cell is going to eat or Ingles, these particular particles, so, what suppose, this is a macrophage and this is a bacteria.

So, what will happen is that as soon as the macrophage will see, it is actually either can recognize these bacteria with the help of the receptors, what is present onto the surface of the macrophages or actually they can be non-receptor mediated identification of these cells as well, they either of that case, this bacteria microphone interaction is actually going to induce the proliferation of the plasma membrane and then eventually what will happen is that the plasma membrane is going to encircle around this particular object.

So, it is actually going to advance around this like this. And then in the third step, what will happen is, these 2 ends are actually going to merge with each other, and eventually, the this particles is going to be internalized into the cell and this is the phenomena which is called as the phagocytosis. What you see actually in this event of phagocytosis is that when the cell will going to phagocytose a particular object, whether it is bacteria or let us beat the cell is going to show you the bacteria or the things which is encircled inside a particular vesicular structures.

And these vesicular structures are called as phagosomes. So, the after the phagocytosis the particles are going to be encircled and inside our membranous structure, which is called as the or vesicles which are called as the phagosomes. So, when you want to study this event, what you see is that the object is going to be encircled by a plasma membrane. So, this means, if I want to study the phagocytosis, what I can do is, I can just simply stain these plasma membrane with a fluorescent dye.

So, what will happen is that the once the object is going to be encircled by the plasma membrane, what I will see is that I will see our object and then I will see a fluorescent signal outside this, which means, that is the object is being phagocytose by the cell. So, this is the philosophy is being used to study the phagocytosis with the help of the fluorescence microscope.

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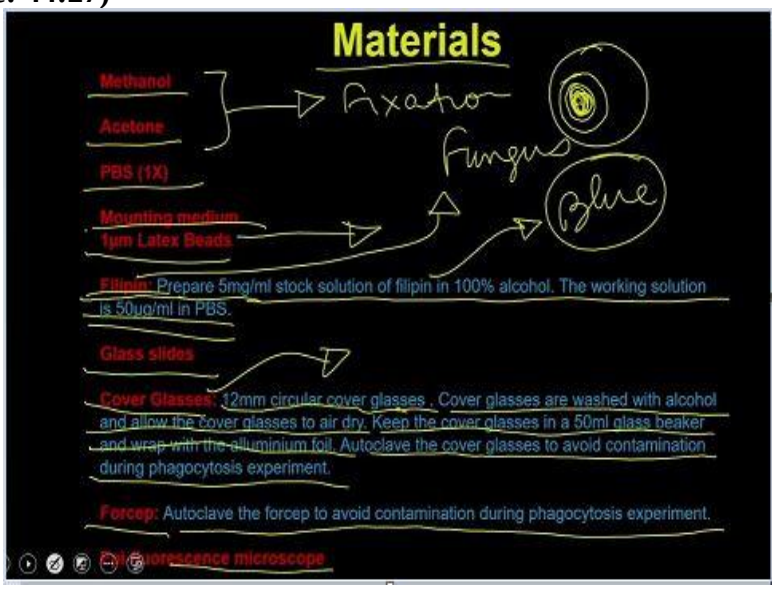

So, for this what are the material required you require the methanol and acetone exactly the same like you require that for fixation of the cells, then you require the PBS then you require the mounting media and you require in 1 micron latex beads because the phagocytosis is being performed by the cell by any particle which is more than 0.5 micron meter which means 4.5 micron meter to 10 micron meter, the cells are going to induce the phagocytosis.

If you have an object which is less than 0.5 micronmeter then the cell is going to engulf that material with the help of a process which is called as pinocytosis then you require a fluorescent dye which is called Filipin. So, Filipin is a actually antibiotic which has been isolated from the fungus and it is actually fluorescent. So, you prepare a 5 mg per ml stock solution of Filipin in 100% alcohol the working solution is 15 microgram per ml in PBS.

And the Filipin is actually going to give you a blue colored fluorescence when it is actually going to interact with the plasma membrane. So, Filipin is a antibiotics isolated from the fungus when it interacts with the cholesterol, it actually gives a blue fluorescence. So, with the help of this, you can be able to stain the plasma membrane and if you if the object is going to be internalized, the plasma membrane is going to encircled around that particular object.

And that is all within the cell you are actually going to see the object encircled by the fluorescently labeled blue color ring. So, that is how it is you can be able to say that this particular object is been phagocytosed then you require the glass slides where you actually going to do a mounting and then you require the cover glasses you require the 12 mm circular cover glasses. So, cover glasses are washed with alcohol and allow the covered glasses to air dry keep the cover glasses in 50 ml class beaker.

And wrapped with the aluminum foil to autoclave the cover glasses to avoid decontamination during the phagocytosis. So, the cover glasses the commercially available covered glasses what you are going to get from the companies are always having 2 problems one they are actually having the glass dust on their surface. So, they are not good for the cells to you know to grow because that actually is causes that injury to these cells.

On the other hand, these glass covered glasses are very, you know very smooth. So, they are been polished by the companies so that they become very, you know, very smooth. So, to you have to remove that particular coating simply by burning these covered glasses by an alcohol. So, what you have to do is you have to dip these covered glasses into the alcohol and then you have to flash these covered glasses into the flame.

So, when you turn when you pass through them with the flames, these polish is going to be removed and then you actually can wipe and clean them. So that the glass dust is also been removed. And ultimately you have to keep them in a beaker and autoclave because, ultimately you are going to use these covered glasses for growing the cells. So there should be no contamination otherwise, the cells will engulf the bacteria and the contamination is also not good for the cells morphology as well as the health as well.

So that is how they are not going to give you the desired results you require the forceps so the forceps are also need to be autoclave. So that you cannot be you should avoid the contaminations and then you require the Epi-fluorescence microscopes.

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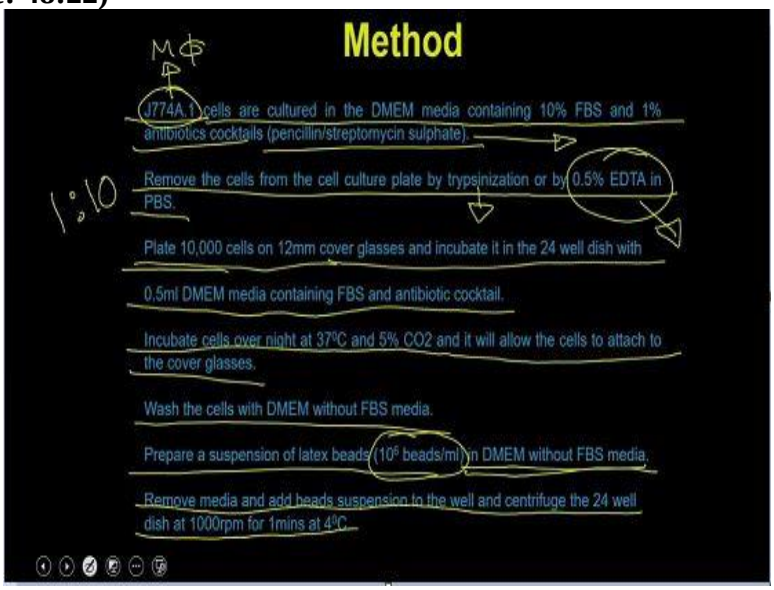

In this particular experiments, we are going to use J774A.1, which is actually our macrofossil line. So J774 cells are cultured in DMEM, and FBS 10% FB and 1% antibiotic cocktails, which actually pencillin streptomycin you might have seen our demo before, where the receiver might have discussed about the different way in which you can be able to prepare these DMEM media

and how you can be able to use them to culture the cells, you remove the cells from the culture plate by trypsinization or by the 0.5% EDTA.

So, you do the trypsinization if you are expecting that the phagocytosis is going to be non-receptor mediated, because when you do a trypsinization it is actually going to remove the all the receptors what is present onto the cell surface. So that actually is not going to allow the cells to go with the receptor mediated phagocytosis. If you have to do a receptor mediated endocytosis then you can use the 5% EDTA and 0. 5% EDTA.

And that actually is going to remove the cells from the dishes you know in a milder way and that is going to remain the receptor intact. You plate the 10,000 cells on 12mm covers glases and incubate it in the 24 well dish with 0.5 ml media containing FBS antibiotic cocktails. So if you do so, the cells are going to adhere to these cover glasses and that so they are ready for phagocytosis experiment. Incubate the cells overnight at 37 degrees Celsius in 5% CO_2 and it will allow the cells to attach to the cover classes, you want the cells with the DMEM.

So that there will be no you know unattached cells so that all the cells are going to be removed, then you prepare the suspension of the latex beads 10 to power 6 latex beads per ml in a DMEM without PBS. So, normally we keep the latex bead versus the sell in a 1 is to 10 ratio which means for every cell you are going to provide the 10 latex bead so that they will be enough latex bead for the cells to you know phagocytosis you prepare the suspension add to latex beads.

And then you are actually going to in the DMEM without FBS media, remove the media and add the latex bead in a well and centrifuge the 24 well dish at 1000 RPM for 1 minutes at 4 degrees Celsius. So if you spin them, all these latex beads are going to be adhere to the on top of the cells. And that is how the phagocytosis will start. And since we are doing this statement at 4 degree, the phagocytosis will start only when you transfer these dish to 37 degrees Celsius.

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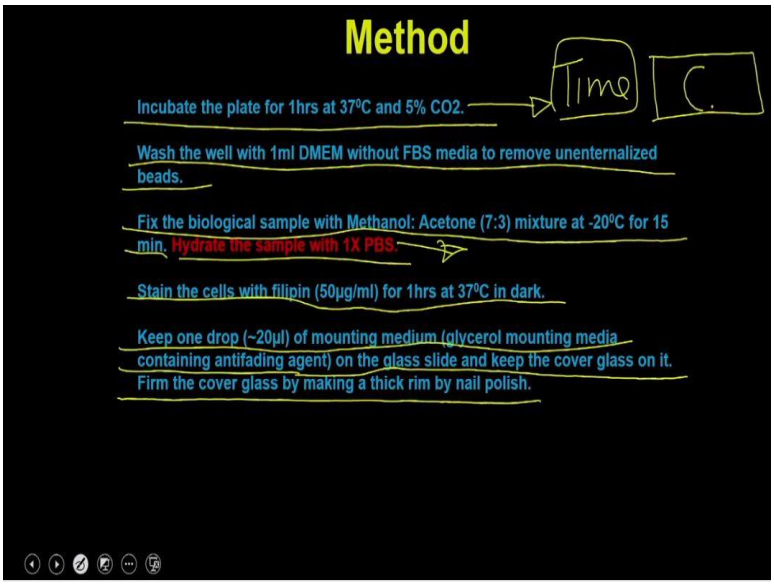

Then incubate the plate for 1 hour 37 degrees Celsius. So 1 hour is good enough for the self to phagocytose the particles. If you want to do a time course then you can actually take out the dishes, you can take out the covers classes at multiple time points. And that is how you can be able to just put them in a fixative solutions and that is how you can be able to stop the process of phagocytosis. So if you want to do a time course, you can do that as well.

Wash the well with 1 ml DMEM without FBS to remove the uninitialized cells and then you fix the biological sample with the methanol acetone mixture at 20 degrees Celsius, you hydrate the sample with 1x PBS so that it will acquire the morphology back then you stain the cells with Filipin for 1 hour at 37 degrees Celsius in dark. As I said you know Filipin is a fluorescent dye so it is actually going to give you the fluorescence and that is how it is actually susceptible for you know the light.

So, you have to do all these statements under the dark or you can actually cover the sample with the aluminum foil keep one drop of mounting media in the glycerol mounting media containing the antifading agent like BPD on the cover slide and keep the covered glasses on it formed the cover glasses by making the thick rim by the nail polish. What is that mean is that when you have a cover slide, you keep the cover glass and then on top of this you have to keep a thick foam of the nail polish so that it is actually going to remain form on that particular place it should not should not move around. Now your sample is ready for visualizations.

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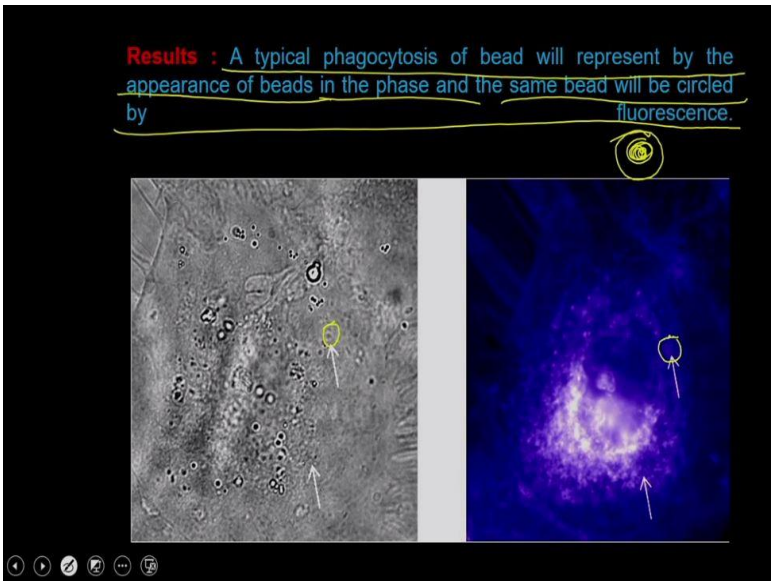

And what you will see is a typical phagocytosis of bead will represented by the appearance of a bead in a face and the same bead with a encircled by a fluorescence which means you are going to see a bead and you are going to see a encircle of the fluorescence for example, in this case, what you see is that there is a bead which has been present onto the cell and the same bead is actually having the blue color fluorescence around it which means these bead is been internalized by the macrophages.

So, this is all about the utilization of the fluorescent microscope there are many more experiments what we can actually discuss and the way in which the fluorescent microscope can be used in one of the approach what we can also do is we can actually studied the interaction of the proteins with the other proteins with the help of the you know the double labeling of the cell. So that actually will say whether these 2 proteins are present when they are in the same compartment whether they are been interacting with each other or not.

So, there are many applications one what when one can do there are many type of experiment one can actually design with the help of the fluorescent microscope. But so with this, I would like to conclude my lecture here. Thank you.