Experimental Biotechnology
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Lecture - 36
Cell Biology Experiments

Hello everybody, this is Dr. Vishal Trivedi from department of biosciences and bioengineering IIT Guwahati. And in this module we were discussing about the cell biology as well as we are discussing about the microscopic tools that is available to perform the different types of experiments. So, now, in today's lecture, we are going to discuss about the different experiments what you can perform with the help of the microscopy.

And I have tried to make a problem like situations so, that you will be understand under what such problem you can be able to utilize the microscopy. So, let us start the discussion about the different experiment what you can perform with the help of the different microscopy techniques.

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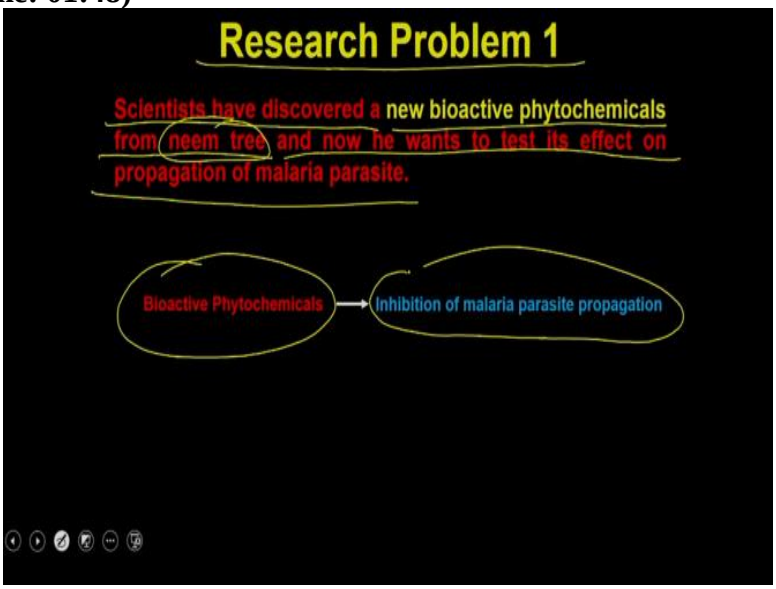

So, our research problem 1 is very simple that scientists have discovered a new bioactive phytochemicals from the neem tree. So, you know the neem tree and now, he wants to test its effect on to the propagation of the malaria parasite. So, what he wants to ask is that he has actually isolated a new bioactive phytochemicals and now, he wants to test whether it is inhibiting the propagation of the malaria parasite or not?

So, you know that the malaria parasite caused a disease called malaria. And if you go with the little background, you know that the malaria parasite requires the 2 hosts, one is invertebrate host and the other is vertebrate host. So, in invertebrate host, you have the mosquito which actually binds to the other vertebrate host, for example, the humans, and that is how it actually goes from one body to another body.

And within the invertebrate host, they have a complete life cycle through which the gametes are fusing with each other to produce the ovum. And that is all they are actually producing the merozoites and then merozoites are being so, that is how they are producing the sporozoites and these sporozoites are actually been injected by the mosquito to the human being, and these sporozoites actually goes into the liver.

And then it completes its lifecycle within the liver to generate the merozoites and these merozoites then infects the RBC's to form the different stages and all the stages what is present in the RBC's are called as the anopheles life cycles.

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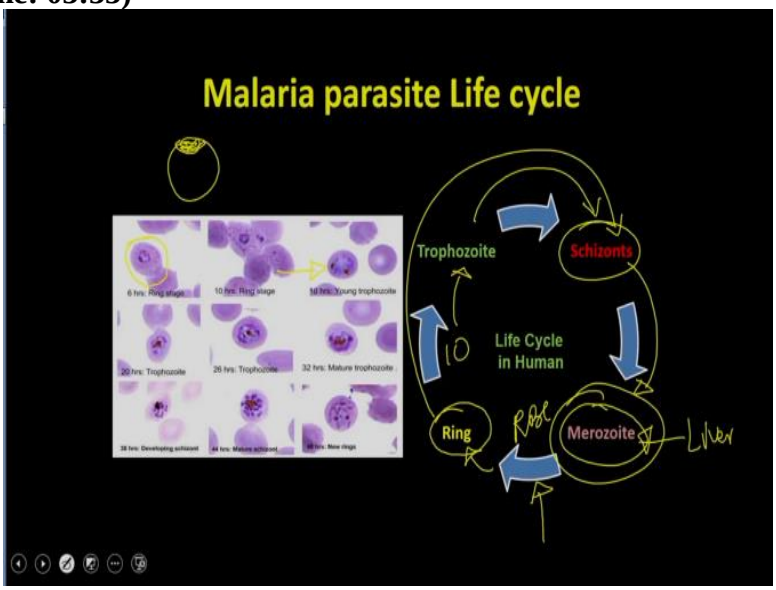

So, within the anopheles lifecycle, you have the different stages like as I said, you know, when merozoites are coming out from the liver, you are getting the merozoites and these merozoites are actually infecting the RBC's and then it is causing it is forming the first stage which is called as the ring stage. So, this is the typical ring stage what you see after as soon as the mirozoites enter into the RBC's.

So, why it is called as ring stage because it is having a ring like appearances where the nucleus is on the one corner and then the cytosol is distributed. And then ring stage is getting converted into trophozoite stage and then trophozoite is actually converting into the schizonts and the schizonts are actually releasing the new merozoites and these new merozoites are again infecting the new RBC's.

So, if you see the life cycle, what you will see is that the merozoites are infecting the RBC's and these RBC's are then forming the ring stage and after the 10 hours the ring stage is getting converted into trophozoite stage and then from trophozoite it is actually causing the production of the schizonts. And then from the schizonts it is actually again releasing the so, once the RBC's containing the schizonts is going to bust it is actually going to release these merozoites.

And then these merozoites are actually going to infect the new series of RBC's. So, what we really want to know is that if we actually treat the parasite or if we treat this particular culture with the phytochemicals, so, whether it is actually going to complete its lifecycle or not.

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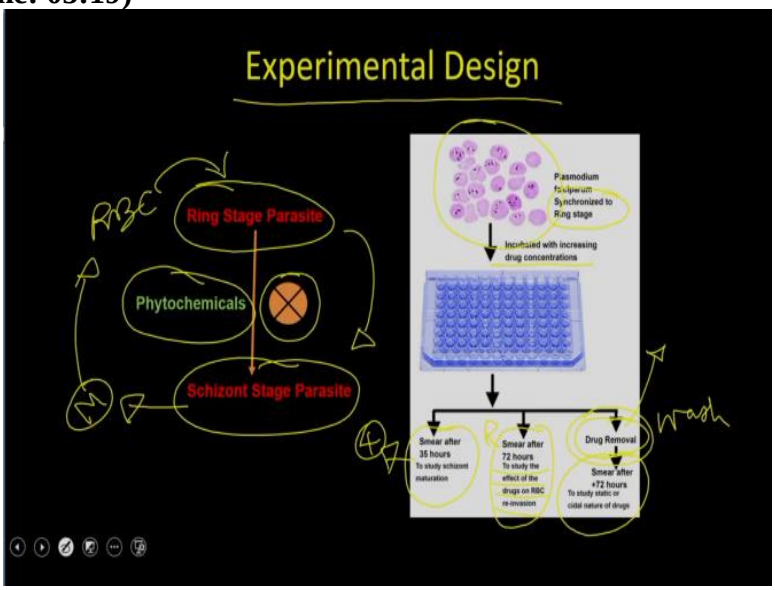

So, experimental design is very simple the experimental design is that you can actually take the ring stage parasites, and then you incubate that with the phytochemicals and you ask whether it is inhibiting the propagation of or the transformation of the ring stage parasite to reach to the schizonts stage parasites. So, typically, what you have to do is you have to just first produce the ring infected RBC's with the help of the synchronizations.

And then you incubate that with the help of the increasing concentration of the phytochemical or the test molecules. And then you incubate that for some time and then after that you actually are going to make a smear after 34 hours to see whether the ring is been mature to give you the schizont stage, because under the normal circumstances, it is actually going to complete its lifecycle and that is how, it is actually going to give you the schizonts within the 35 hours.

If you want to study the reinvasions, then you again make a smear after 72 hours and that actually is going to tell you whether your compound is also having an effect on the reinvasions; reinvasion means, the schizonts are going to release the merozoites and these merozoites are whether infecting the new RBC's or not because that is how it is actually going to give you the new ring stage.

You can also ask whether the compound is the parasitotatic or the parasicidal which means whether the compound is killing the parasite or whether it is simply stopping the growth of the parasites that you can do simply by drug removal. So, what you can do is you can treat the parasite for the drug molecules or the test molecule for some time, and then after that, you actually can remove the parasite and keep it into the fresh media and let them to propagate.

So, if the parasite is dead, it is not going to propagate into the fresh media, but if it is only, you know, stopping its growth, which means it is still be live then it will start making the growth. So, that is what you have to do when you just remove the drug, you wash the parasite culture, and then you put it into a new media and then you if you prepare the smear after 72 hours.

If you see the viable parasites, then actually it is going to say that compound what you are testing is the parasitotatic in nature means it is actually stopping the growth of the parasite, but it is not killing the parasite. So, these are the 3 questions one can ask with the help of this microscopy based assays where when you can say whether the compound is killing the parasite or not, whether the compound is inhibiting the reinvasion event or not and whether the compound is parasitotatic or the parasicidal.

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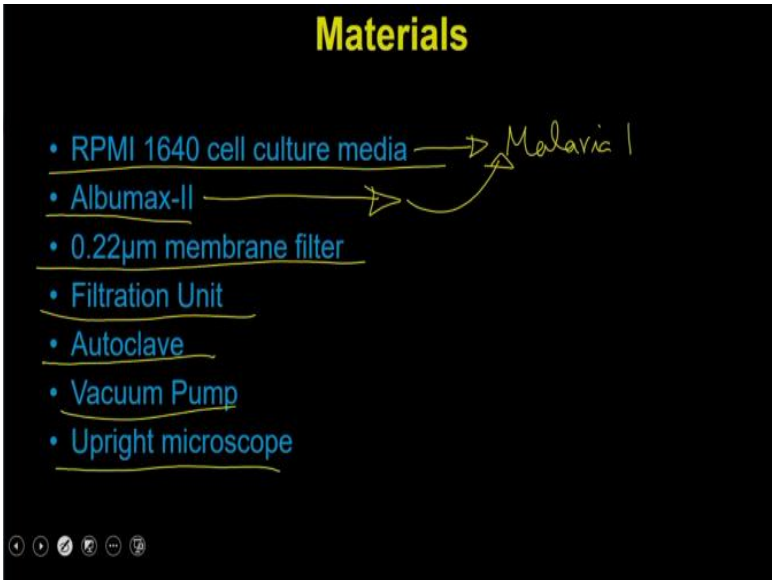

So, for to performing these experiments, you require the media like the RPMI 1640 cell culture media, which we require to culture the malaria parasite then you require the albumax 2 which is actually a powder which actually acquire for propagation of the malaria parasite, you require the 0.22 micron filters, you require the filtration units to prepare the media you require the autoclave, you acquire the vacuum pumps.

And then you required upright microscope so, that you can be able to visualize the parasites after the different stages like if you can visualize after 34, 35 hours or 72 hours or after the removal of the drug, you can be able to visualize the microscope visualize the parasite with the help of the upright microscopes.

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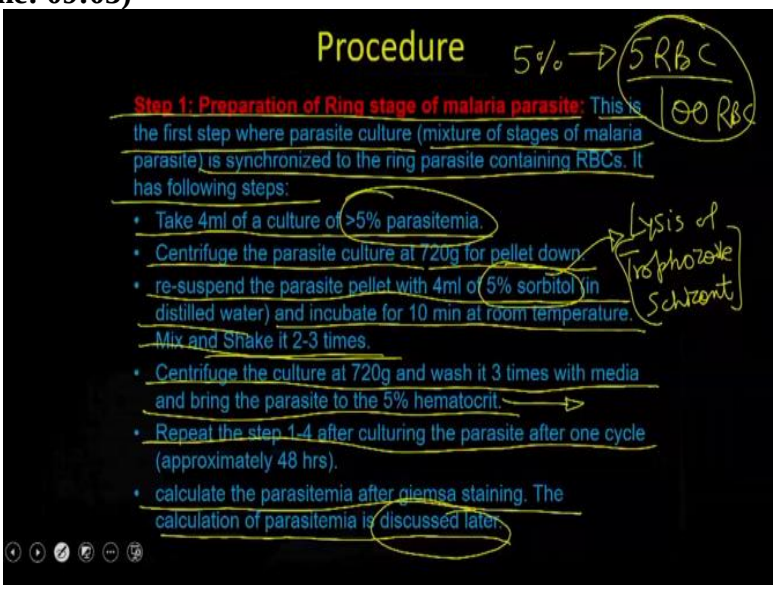

So, in the step 1, you have to prepare the ring stage parasites. So, this is been the first step where the parasite culture the mixture of different stages of the parasite is synchronized to the

ring parasite containing RBC's it has the following steps. So, the first step is that you have to prepare the ring infected RBC's so, that you can be able to then incubate those ring infected RBC's with the test molecules and say whether the ring is getting matured to give you the schizont after 35 hours or not.

So, for that, you have to prepare the ring stage and that you have to do with the help of the synchronizations. So, what you do is you take the 4 ml of a culture which is where you have the parasitemia of 5%. So what is mean by the 5% parasitemia is that the 5 RBC's are infected out of the 100 RBC which you are going to count which means that is the 5% parasitemia.

So, you centrifuge the parasite culture at 72 g for to pellet down the RBC's and then you resuspend RBC pellet with 4 ml of 5% sorbital in distilled water and incubate for 10 minutes at room temperature and mix and shake it for 2, 3 times. So, when you incubate this parasite culture with a 5% sorbitol, what will happen is that it is actually going to induce the lysis of the trophozoites, as well as the schizonts containing a parasite.

So, it is actually going to lyse all the RBC's which actually contains the trophozoites or the schizonts, but it is actually not going to affect the RBC's which contains the rings parasites. So, then you once you shake it for 2, 3 minutes, it is actually going to perform its actions and then you centrifuge the culture at 72 g, wash it for 3 times with media and bring the parasite to the 5% hematocrit.

So, you know that the 5% hematocrit means that the 5% of the RBC's out of the 100% volume which means the only the pack volume of RBC is going to be 5%. The normal blood has a hematocrit of 50% which means the 50% is the RBC component and the 50% is the plasma. So, in this case, we are taking only the 5% hematocrit. You repeat this you know synchronization steps for 2, 3 times.

Because you know, first time you when you do the major chunk of the schizonts and the trophozoites are going to be lysed and then you may still have some schizonts and trophozoites left. So, then your rings are going to be formed and you may have also the rings of the different stages as well because you can have the ring which is actually going to be formed after 2 hours you can have the ring which has actually been off for 8 hours.

So what will happen is in within another 4 or 5 hours or 10 hours you will see again you will start seeing the trophozoites to make it very, very precisely the synchronized culture what you can do is you can do the synchronization with the help of this D-sorbitol multiple rounds like first time you have done then you again culture it for another 48 hours, then again you do it.

So, if you repeat 2, 3 times, you are actually going to bring only the ring containing parasites and that is good enough for performing this assay, then you calculate the parasitemia after the giemsa stain the calculation of the parasitemia, we are going to discuss so parasitemia you do not have to worry parasitemia means the number of RBC's present in the 100 ml RBC's means number of parasite containing RBC's out of the 100 RBC's that is called as the parasitemia and we are going to discuss about how to calculate a parasitemia in our subsequent slides.

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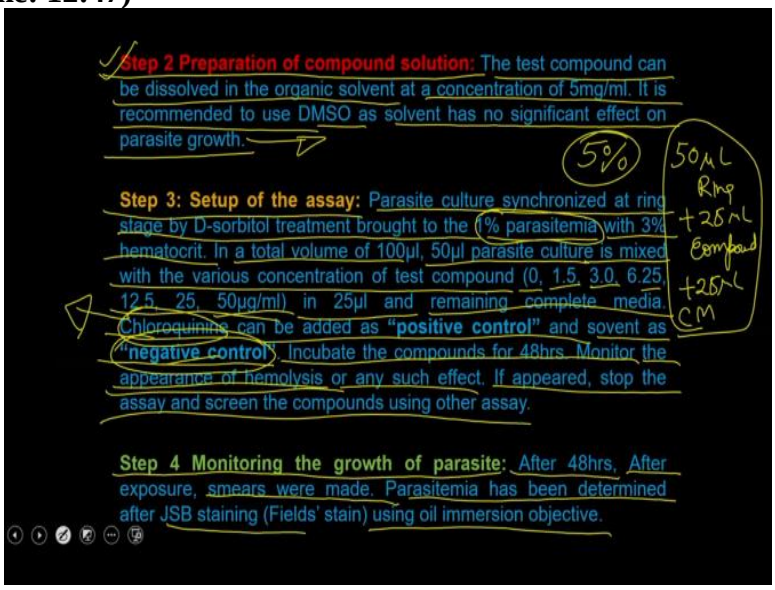

So, now, once you have prepared the ring containing RBC's then you have to do a preparation of the compound solutions for the test compound can be dissolved in the organic solvent at a concentration of 5 mg per ml. It is recommended to use DMSO as a solvent as it has no significant effect on to the parasite growth. So, if it is a phytochemicals or if it is a water soluble compound then you do not need to dissolve it into organic solvent you can simply dissolve it into you know the aqueous media.

And then you can just pass through 0.22 micron filters for that it should be filter sterile. Otherwise, what will happen is as soon as you will add the compound the bacteria will start growing. So, that actually is going to interfere with your assay system. And so in the step 2, you are going to prepare the compounds. And then in step 3, you are going to set up the assay for the parasite culture synchronized at ring stage by the D-sorbitol treatment brought to the 1% parasitemia.

So, remember that it was a 5% parasitemia. So, whatever the parasitemia you have, you bring it to the 1% parasitaemia with the 3% hematocrit and in a total volume of 100 microliters you take the 50 microliters of parasite culture and it is mixed with the various concentration of the test compound like 0, 1.5, 3.0, 6.25 so, it is a serial dilution of up to 50 microgram per ml of a compound in 25 microliter and in the remaining you can add the complete media.

So, in a recipe what you have is you have a 50 microliters of you know the ring containing cells, then you actually add the 25 microliter of the compound which is actually going to be of different concentrations. And then you add the 20 microliter, 25 microliters of the complete media and that is actually you going to incubate it into the incubators and let them to you know, grow for another 48 hours in the presence of the compounds.

Since you are doing all these experiments you have to add the chloroquinine, which is actually an anti malarial compound as a positive control and you can add the solvent as a negative control. So, negative control is like whatever the solvent you are using, if it is you know aqueous solvents like PBS or buffer or whatever then you can add the buffers, if it is a DMSO, then you have to add the DMSO and that will be a negative control and then you can add the chloroquinine also.

So, that it will tell you that whether the assay was working or not, because the chloroquinine is eventually going to kill the parasites, so, it is actually going to work as a positive control, incubate the compound for 48 hours, then you monitor the appearances of the hemolysis or any other such effect, if it there will be an hemolysis because what happened is sometime you whatever the test compound you add, it actually does not have any parasitotatic effect, or it is does not have a parasicidal effect.

But instead of that, it actually no, lyse the cells. And that is how actually it is going to interfere with your assay system. So if that happens, then you because if the RBC's are being lysed, then there is no way that the ring are going to be propagate to give you schizont and then schizont is going to give you the merazoite and then it is actually going to do a reinvasions.

So, if you have any hemolysis, if you there will be any lysis of the RBC, then you have to stop the reactions. And then you have to investigate which component of your assay mixture is actually giving you the hemolysis if you can manage, because there is some time what happened is the asmo morality of the different compound is also interfering with the asmo molarity of the complete reactions. And that is how it is actually causing the hemolysis.

So, sometimes you may have to change your buffer systems and all that to take care of that. So that is how you are to actually use the stop the assay, but otherwise, if you do not see any hemolysis, then you can go on to the next step and you can monitor the growth of the parasite. So in the step 4, you are going to monitor the step of the growth of the parasites. So, after the 48 hours, you are going to make a smear and the parasitemia has to be determined after the JSB staining so, using an oil immersion objective.

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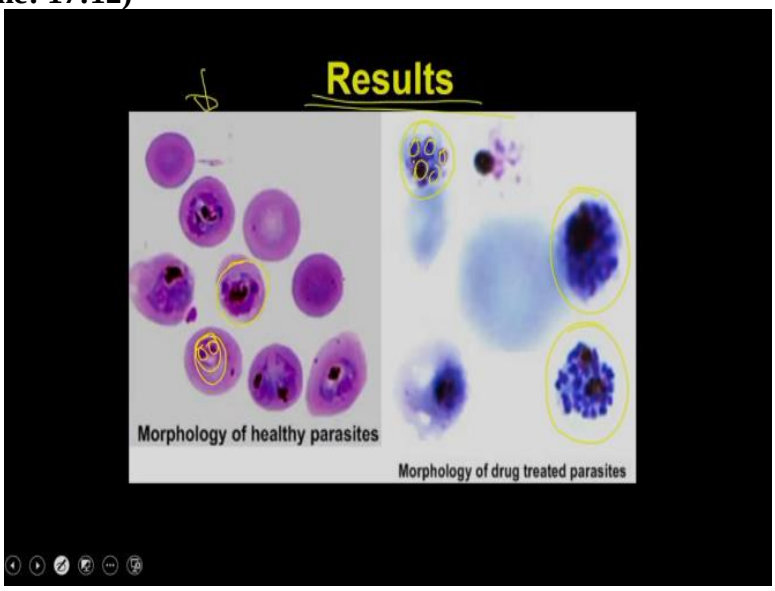

So, and these are the results what you are going to see. So, what you see is these are the healthy parasites. So, if there will be no inhibition of the compound then what you are going to see is that ring is actually getting converted into the all other stages of the parasites like

you will see the trophozoite and schizont and all that kind of thing. But if the ring is not been able to convert into the schizonts or even if it get converted into schizont.

But the schizont is been dead, then what you will see is actually RBC which is actually going to have the fragmented cells like you are not going to see a clear nuclear membrane and you will not be able to see a clear cytosol because for example, in this case, you see that it has a very you know discrete cell and it has a 2 nucleus, and it is very clean and clear, but in this case all these cells are actually fragmented.

So, these are actually the appearances what you will see when the parasite is dead actually, because that is how it is actually does not have the nuclear membrane and the cytosol is also not very clear. So, that is how it will say that whether the assay is working or not, but if you want to calculate the growth of the parasite, then what you have to do is you have to count these cells under the microscope.

So, you have to observe these cells under the 100 x of magnifications. And then you have to keep counting how many number of RBC's are ring containing how many RBC's are of trophozoite and how many are schizonts and then you have to plot that and using that particular data you will be able to determine the parasitemia as well as whether the compounds are inhibiting or not.

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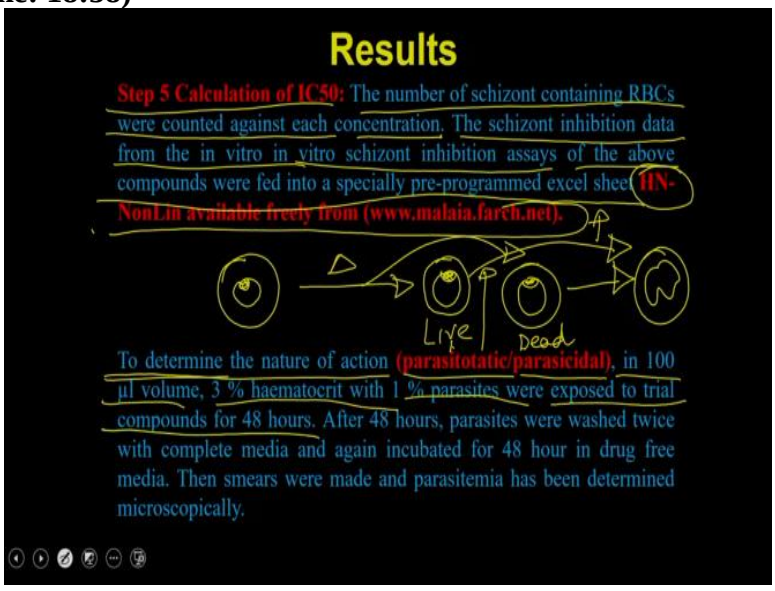

So, in the step 5, using that you can be able to calculate the IC50 which means the inhibitory concentration 50 of that compound. So, the number of schizont containing RBC's were

counted against each concentrations the schizont inhibition data from that invitro schizont inhibition assays of the above compounds were fed into a specially pre programmed excel sheets.

So, you can actually be able to calculate using this excel sheet which has been available from this particular site. So if you put all that schizont containing cells and put it into this particular excel sheet, you know, automatically it is going to plot the curve and it will going to tell you the IC50 as well as the IC90, and all other kinds of parameters. You can add the chloroquinine as a positive control and that actually is going to give you the confidence that the assay was working.

And there is no you know, the flaws or there is no issue with the setup of the assay itself to determine the nature of the action, for example, the parasitotatic or the parasicidal, parasitotatic parasicidal operation means that you have you know, our ring containing parasite. So, what you will do is you will add the compound. So, if you add the compound what will happen is that it is actually going to seize the you know the growth of that, particular parasites.

So, whether it is seizing because the parasite is you know not getting enough nutrition and in your know the compound is somewhat interfering with the biochemical pathways and therefore, it is decided that let us you know reduce the metabolic activities and remain as you know dormant stage. So, in that case the parasite is going to be remained life, but it is not going to grow actually, because for growth it requires all those metabolic pathways to be in an activated state.

The other condition is that it is actually containing you know, converting the parasite into a ring containing parasite, but these rings are actually the dead. So, if they are dead, then they are not going to give you the schizont whatever you do actually, but they are live and if you remove the compound, which is then they are actually going to give you the schizont, which means they can be able to complete their life cycle.

So, if they are being able to you know the life and they will be able to complete then the compound is going to be called as the parasitotatic which means it is actually only inhibiting the growth of the parasite, but if it is converting the parasite to a dead parasite, then the dead

parasite will not going to complete it lifecycle and that is how it is actually going to be called as the parasicidal which means it is going to kill the parasite.

So, to determine that what you have to do is in 100 microliters volume 3% hematocrit with 1% parasitemia were exposed to the trial compound for 48 hours after 48 hours the parasite were washed twice with complete media so that you can be able to remove the compounds and then you incubate for other 48 hours in a drug free media. So, once you keep it in a drug free media, they are actually going to be free to you know grow if they are live, they will grow if they are dead, they are not going to grow.

So, then you are going to prepare a smear and the parasitemia can be determined with the microscopically even as we discussed like you prepare a smear, then you count the you know the 1000 cells, which means the 10 fields actually and if you count the 1000 cells, it will actually going to give you the statistically significant how many number of infected RBC's are present and that way you are actually going to determine the parasitemia.

So, this is all about one problem where we have used the light microscopy and we have actually determine the anti malarial activity of a test compound you can be able to modulate the spines of assays and you can be able to even use it for some other application. For example, even if we see what we have discussed, we have discussed about the malaria parasite, but if you want to change the conditions.

And you want to utilize the microscopes, you can be able to change it in accordingly. And that is how you can be able to utilize it even for screening the compound for other assays as well. For example, you can use the even slightly derived version and you can be used to microscopy to measure whether, you know for even for cancer cells and all other kinds of cells, whether the cells are growing or not.

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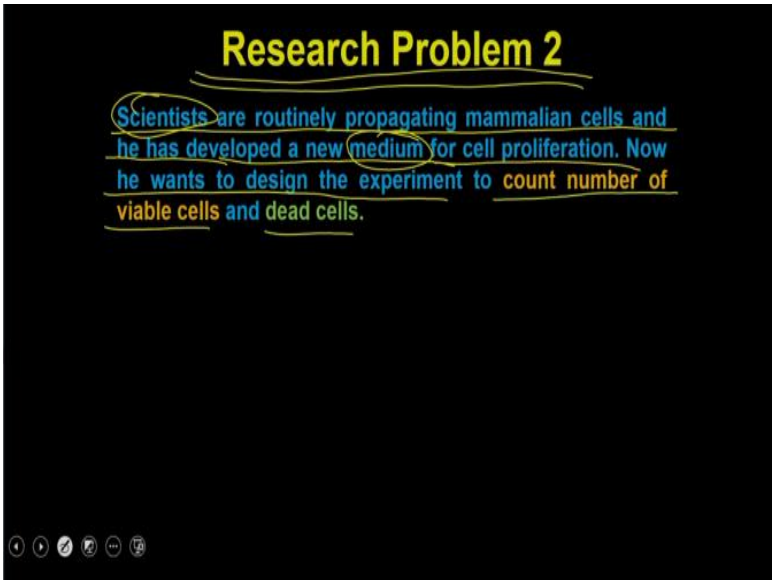

Now, we will discuss about research problem 2 so, in the research problem 2, the scientists are routinely propagating mammalian cells and now, what the scientist has done, it has developed a new medium, which means it he has developed a new media for cell proliferations. Now, what he wants, he wants to design an experiment to count the number of viable cells and number of dead cells which means, he has developed a new media.

And now, what he wants is he since he wants to test whether this media is good enough, as it was already been established media compared to the established media and whether the number of live or the dead cells are more or less in the case of when we they are using this particular new media for propagations.

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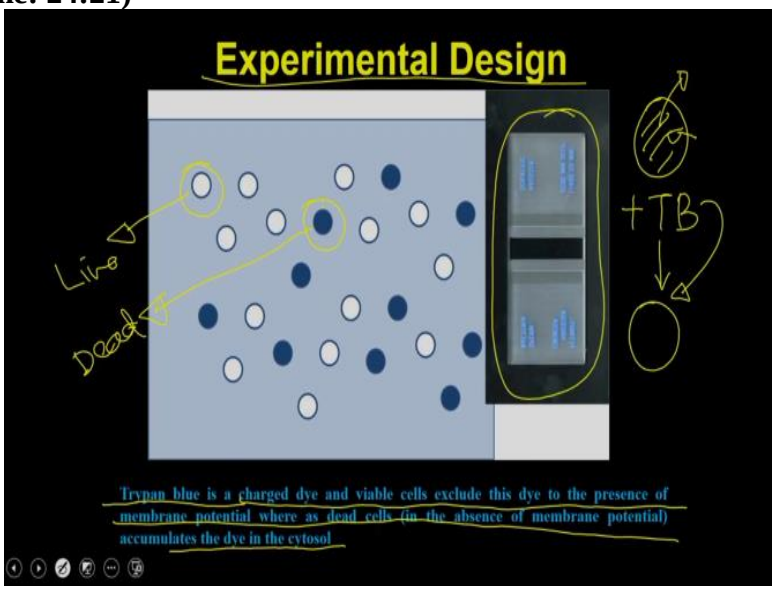

So, experimental design is very simple you are going to use a dye which is called as the trypan blue so, trypan blue is a charged dye. And the viable cells exclude this dye to the

presence of the membrane potential whereas dead cell are actually going to accumulate the dye in the cytosol. So, what happen is you take the cells or you take the culture actually, and then if you add the trypan blue what will happen is the trypan blue is actually going to be excluded by the cell because trypan blue is a charged dye.

So it requires some receptor or some other active processes through which trypan blue can be taken up by the cell, but if the cell is dead, because the cell is maintaining a, you know, a potential across the membrane and the dye has to neutralize that potential, then only the dye can be able to enter, but if the cell is dead, that trypan blue is actually going to be entered into the cell and it is actually going to make the cell as the blue color.

So, that is what you have to do, if you take the whole cell populations, and if you stain it with the trypan blue what you will see is that the some cells which have not taken up the dye, and the some cells, which are actually appeared blue, so, these are actually the live cells, because they are actually opposing the entry of the dye into the cell, because they have active membrane potential.

And that membrane potential is opposing the entry of the compound compared to this, this is a dead cell and that dead cell does not have the required electrode, the membrane potential and that is how, it is actually going to allow the entry of the dye and the dye is actually going to accumulate into the cells, this is the hemocytometer which actually you can use to count these cells with the help of the microscopy.

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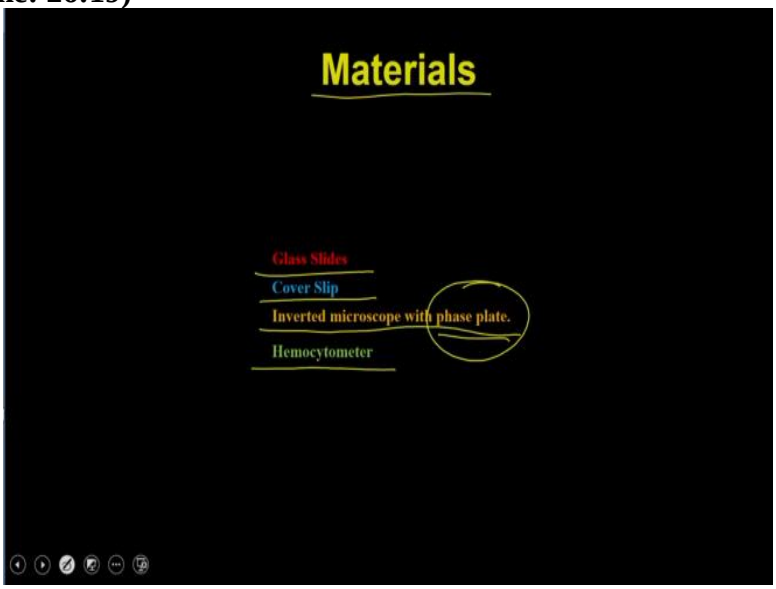

So, the material what you require the material what you require is a glass slide, you require a coverslip, you require an inverted microscope with a phase contrast provision, like you require a phase plate and then you require a hemocytometer.

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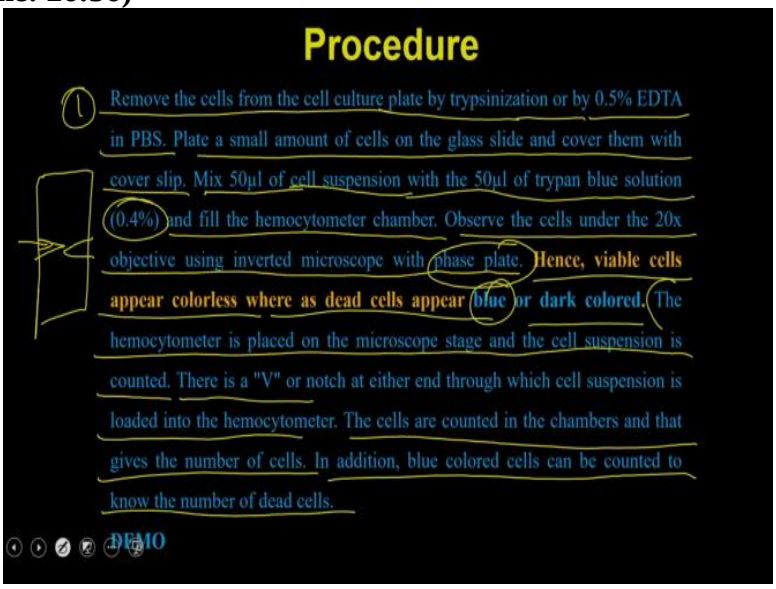

These are the multiple steps so, in the step 1, what you have to do is you have to remove the cell from the cell culture plate either by the trypsinization or by the 0.5% EDTA. So, this is the step 1, and then you plate the small amount of cells on a glass slide and covered them with a coverslip then what you do is you take the mix 50 microliters of cell suspension with the 50 microliters of trypan blue solutions.

So, 0.5, 0.4% trypan blue solution is already available from the different vendors that you can buy, and then you fill the hemocytometer chambers, observe the cell under the 20x objective using the inverted microscope with the phase plate. So, viable cells appears colorless, whereas the dead cell appear blue or the dark colored, the hemocytometer is placed on the microscope stage and the cell suspension is counted, there is a V color notch.

So, in the hemocytometer, what you have is in the centre you have a V color notch. So, on this V color notch you can actually you know, through with that you V color notch, you can be able to you know, you can be able to load the hemocytometer with the cells, and the cells are counted in the chamber and that gives the number of cells in addition, the blue colored cell can be counted to know that the number of dead cells.

So in this particular procedure, what you have to do is the first you have to trypsinize the cells. So, when you add the trypsin enzyme, or you can use the EDTA, it is actually either going to chew up all the receptors, what the cells are using to stick to the dishes, or it is actually going to destroy the calcium. So, whatever the mechanism, the cells are going to come off from the dish. And then what you can do is you can just mix the 50 microliters of the cell with the 50 microliters of trypan blue.

And then you load that onto the hemocytometer and then you put it under the inverted microscope and then you can actually visualize the cells within the chamber. And you can be able to, you know, count the number of blue cells and the number of colorless cells and that is how you can be able to count the number of dead cells and number of viable cells. So, this is all about a theoretical understanding of this process.

Let me take you to my lab and we are going to show you all these procedures and because the hemocytometer is a very, very, you know, if you very clearly see the inside the structure, it actually has a different chambers, and then you have to do a counting in these different chambers. So, that you will be able to count the number of cells and number of cells and then you ultimately you can be able to even calculate the concentration of the cell in per microliter or number of cells per ml as well.

Because that information is required if you want to plate a specific number of cells for an experiment. For example, if you remember when we were doing the, you know the when we were discussing about the phagocytosis experiments, the last time we said that you have to plate the 10,000 cells. So, if you will want to plate the 10,000 cells the first thing what you have to do is you have to put the cells into the hemocytometer you have to count.

And then you have to convert that value into the concentration like 10 to power 6 per ml or 10 to power 8 per ml or so on. And then accordingly you have to dilute and calculate that how many how much microliter of the cell suspension I should take so that it will give me the 10,000 cells per well. So, that is also true for and all other assays like MDT assay and you know, all other kind of assay what you do in your lab where you have to do a counting. So, let us understand how you can do the cell counting and how you can be able to determine what are the numbers of viable cells you have and what are the dead cells you have.

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Hello everyone, in this video, we will show how to subculture the cells and count the cells. First we have to remove the remaining media. Then trypsinize the cells and we will count the cells and stain it. Now I will show how to do trpsinization. Now, I am going to add the trypsin to detach the cells. After cells are detached, we have taken into clean falcon then we have to centrifuge these cells.

As these cells are very delicate, we have to centrifuge at 1500 rpm for 2 minutes. Now, we have to remove the supernatant and resuspend the cells in fresh media. After resuspension, we have to count the cells so I am going to take 20 microliter of the cell suspension and mix with the 20 microliter of trypan blue and count under neubauer chamber. Before counting, we have to see how a counting chamber or a hemocytometer look like.

This is a typical hemocytometer also called as neubauer chamber, which contains this squares in upper side and lower side with each square having depth of 1 mm and area of 00.25 millimetre square. Now, I am going to put a coverslip on this chamber. Then, I will add slowly cell suspension through capillary action it will spread all over the squares. So, we checked how many sets are there in all the squares.

Now, how to count the cells? So, here a typical neubauer chamber which contains squares 5 squares. So, we have to count cells in these squares. So, each square is an area of 0.0025 millimetre square and total small squares 16. So, total area of this whole square is 0.04 millimetre square. So, the depth of this each well is 0.1 millimetre. So, what is the volume 0.04 into 0.1 so, that is total 0.004 millimetre cube or 0.004 microliter.

So, say we have counted the cells in each well say this is A, B, C, D here we have 100, here we have 150, here we have 110, here we have 100. So, the total cells we have to take average that means, 100 + 150 + 110 + 100 / 4, total 4 squares we are counting the average is 115. So, 115 cells in 0.004 microliter volume, so, how many cells per 1 ml so, that we can calculate simply by 0.004 into 1000 that will give value cells per ml.

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So, in this experiment the student have discussed different steps related to the cell counting and how you can be able to utilize the microscopes to determine the viable as well as the dead cells, they also have discussed how to calculate the concentration of your cell so, that you can

be able to use that information to you know put, you know, you can be able to use that information to put the cells as per the your requirements.

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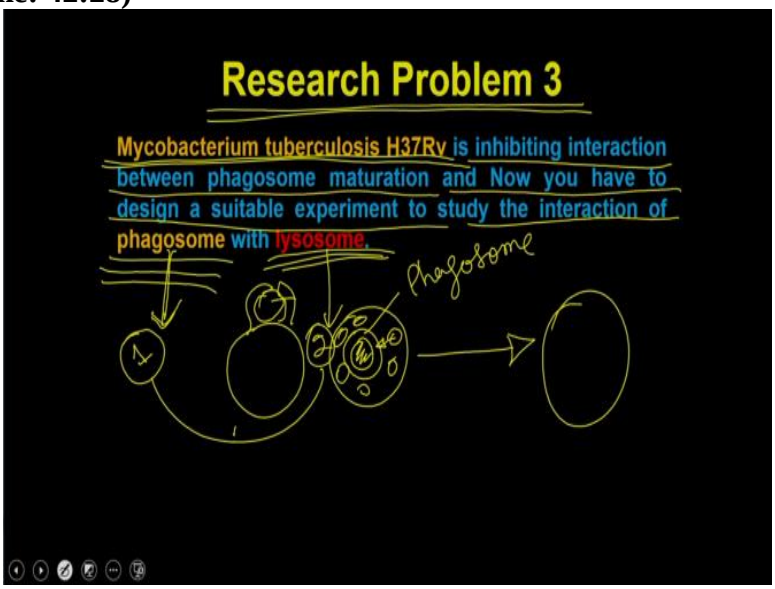

Now, let us move on to the next problems. So, the next research problem is that mycobacterium tuberculosis H37Rv is inhibiting the interaction between the phagosome maturation and now you have to design a suitable experiment to study the interaction of the phagosomes with the lysosomes. If you remember, when we were discussing about the phagocytosis in our previous lecture, we said that the phagosome is formed when a particle is being taken up by the cell through phagocytosis.

So, once a cell is being taken up a particle, like for example, a bacteria, in this case, the mycobacterium tuberculosis, this particular particle is going to be phagocytose with the help of the pseudopodia. And then ultimately, it is going to be internalized. So in the internalize thing, what you have is you this is a bacteria what you have, and it is been internalized by a membranous structure. And this structure is actually been called as the phagosome.

And then eventually what happened is that you have the lysosomes in the cell, so all the immune cells have the lysosomes. So, then these lysosomes actually fuses with these phagosome. And this is a very, very complicated and complex process through which the phagosome actually interacts with the lysosomes and eventually developed and you know, the lysosomes fused, and then when the lysosome is fused, it actually causes the release of its content.

And that is how the phagosome is lysosome is actually going to release its content and that is how lysosome is going to digest this material. So, lysosome is you know lysosomes, inner pH is around 2 and it also contains different types of enzymes like hydrolysis and proteases, and all those kinds of enzymes. So, all these enzymes are actually processing the cell, the bacteria, whatever the immune cells have been engulfed, and that is how it actually degrades the bacteria.

But what happened in the case of mycobacterium tuberculosis, it actually interferes in this whole process and eventually survived inside the macrophages and that is how it actually propagate within the immune cells. And that is how they will be able to rescued or survived from the action of the immune cells. So, that is what we supposed to do, if we have to suppose to design an experiment to study the interaction of the phagosome with the lysosomes.

So, in this experiment, what you have to do is you have to first you know, form the lysosomes first you have to form the phagosomes and then you have to form the lysosomes and then you have to mix them together and then you have to ask them, whether they are actually fusing or not in the presence of tuberculosis or bacteria or not, or suppose you give the phagocytosis the mycobacterium tuberculosis bacteria itself for phagocytosis and then you study these interactions.

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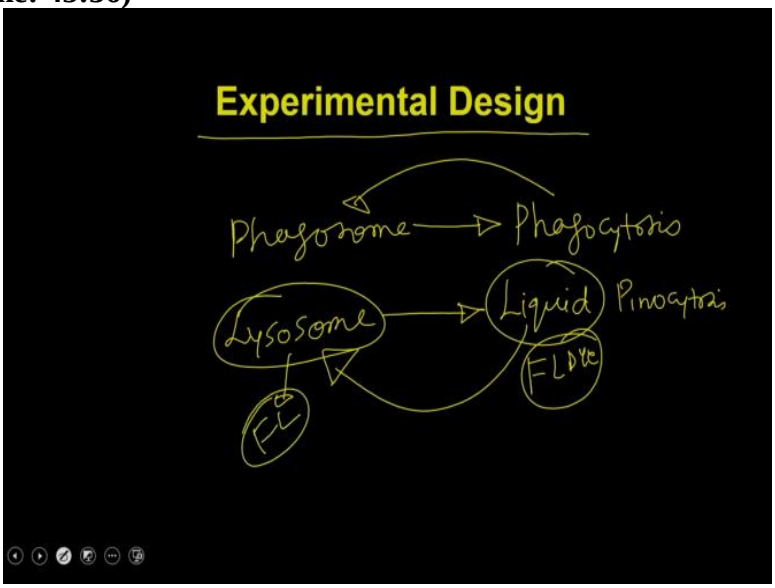

So, we have already discussed about the experimental design, where you have to first you know, form the phagosomes, then you have to also form the lysosomes. And how you are

going to do a phagosomes, if you are going to form the phagosome what you can do is, you can simply allow the cell to do phagocytosis and phagocytosis if you do it is actually going to form the phagosomes how you can be able to generate the lysosomes what you can do is you can ask a cell to drink some liquid.

For example, you can just go with the process of pinocytosis and if you do so, whatever you liquid you will give that will enter into the lysosomes. So, for example, if I take a fluorescent dye and if I add that dye to the cell culture media, eventually what will happen is the lysosomes whatever is going to be formed are actually going to be fluorescently labeled because this liquid what we are taking is actually going to be filled into the lysosomes.

Once these 2 are ready, then you can mix them and ask whether they are fusing with each other or not. Because if they mycobacterium has any effect or it is actually having some factors which are interfering into this, it is actually going to, it will not allow the phagosome to interact with lysosomes.

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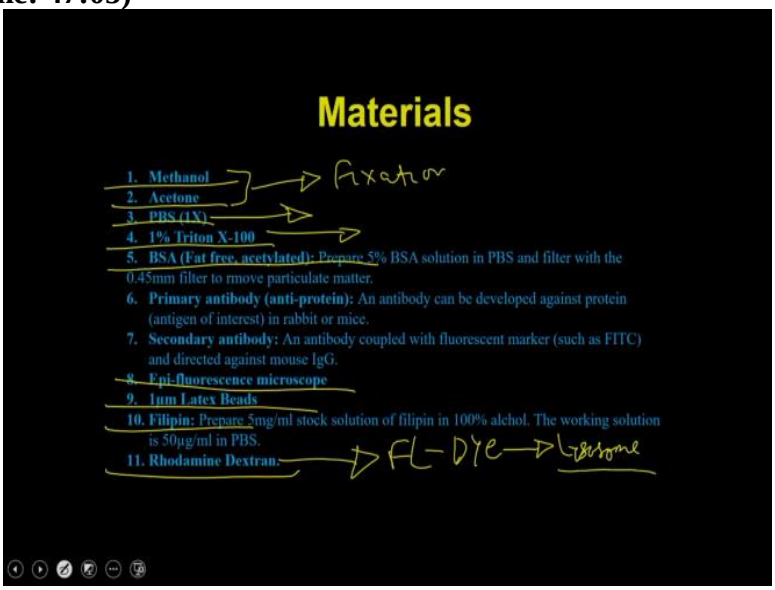

So, the material what you require? You require the methanol and acetone that is you require for fixation, you require the PBS that is only for washing purpose then you require the triton X 100 that is for permutations you require the BSA, you require the you know epi-fluorescence microscopes, you require the 1 micron latex beads the required the filipins and then you require the rhodamine dextran so, rhodamine dextran is a fluorescent dye, which actually going to allow you to form the lysosomes.

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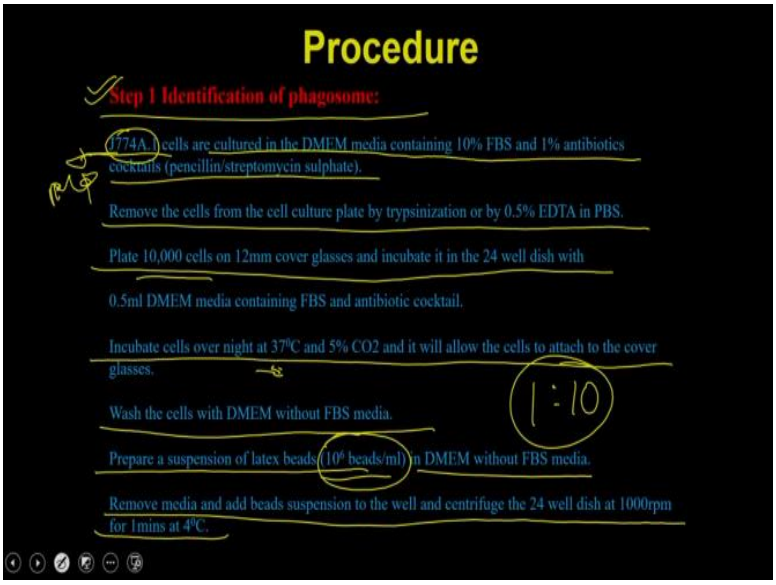

So, the procedure what we require the procedure is that in the step 1 you are going to do the identification of the phagosome or the you are going to prepare the phagosomes which you can as per you know provided by the your own material. So, what you can do is you can take the J774 these are the macrophages cells, so, these are the immune cells, which you can use for phagocytosis.

So, these are the macrophages cells are cultured in DMEM Media containing 10% FBS and 1% cocktails. Then you remove the cells from the cell plate by trpsinization or EDTA. You plate the 10,000 cells on cover glasses and incubate in a 24 well dish you then you incubate the cells overnight, and allow the cells to attach to the cover glasses. Then you wash the cells with DMEM then you prepare a suspension of the latex beads like 10 to power 6 latex beads per ml in a DMEM media.

So, what you have to do is you have to maintain a ratio of 1 is to 10 in terms of the number of cells what you have taken and the number of beads what you have taken. So you are going to provide the 10 beads for every cells to feed and then you remove the media and add the suspension to the centrifuge. And so basically what you are doing is in the step 1, you are basically doing phagocytosis and that is how it is actually going to eventually form the phagosomes.

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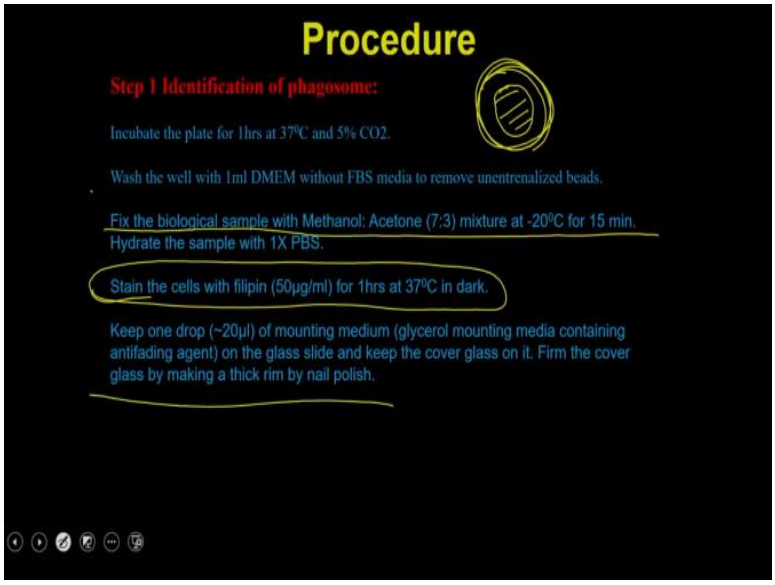

So, these are the, you know, the wash the cells with this and that and then you fix the sample with the acetone and then you stain it with the filipines. So, that actually is going to allow you to identify the phagosome because wherever you will going to see the object being encircled by a blue color fluorescence that is actually the phagosomes and then you mount the cells into you know, and you can be able to identify the phagosomes.

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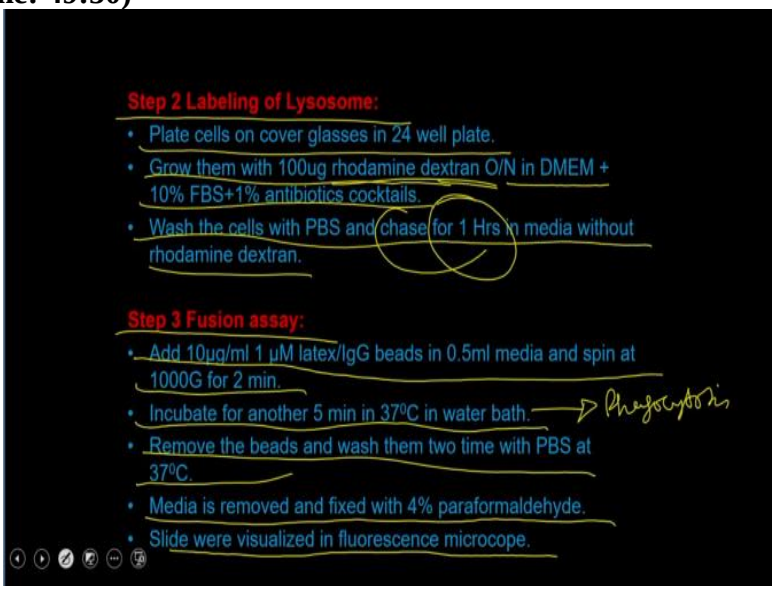

Then in step 2, you are going to do a labeling of the lysosomes. So, what you do is you plate the cells on a cover glasses in 24 well plate grow them with 100 microgram rhodamine dextran overnight in DMEM plus 10% FBS plus antibiotic cocktail. So, what will happen is when you are going to grow the cells in a media which contains the rhodamine dextran so, rhodamine is a fluorescent dye and it is coupled with a dextran. So, dextran is nothing but a polysaccharides.

So, what has happened is the cell will start going to eat these rhodium dextran. So, when it will eat the eventually all the materials end up into the lysosome because you know that primary function of lysosome is to digest the material what you have ingested or the whatever the you know, the material is being ingested by the cells, because ultimately, you have to you know, you have to digest all that material, generate the constituents material like suppose you digest the protein, then it is going to generate the amino acid.

And then these amino acids are going to be supplied, to the cell for its propagations or for nutrition. So, whatever the media you are using, you use the same media you add the rhodamine dextran and that you let them to grow for overnight. And that is how the rhodamine is going to be end up into the lysosomes you wash the cells with PBS, and then you are going to chase the sample for 1 hour.

So, what is mean by chasing is that you remove the rhodamine dextron and then you let that to grow without you know without rhodamine dextran. So, what happened is if you do so, the last drop of the rhodamine dextran will also going to be end up into the lysosomes because eventually everything ends up into the lysosome because that is the way the cell has devise a mechanism so that whatever it drinks or whatever it eats, it ends up into the lysosome so that it will get digested.

And you can be able to use the nutrient coming out of these digestions. Then in the step 3, you are going to set up the fusion assays. So you add the 10 microgram 1 ml latex in 0.5 ml media and spin at 1000 g for 2 minutes, then you incubate for another 5 minutes in water bath. So, when you do that incubation in 5 minutes for water bath, it is actually going to induce the phagocytosis of these beads.

And then you remove the beads and wash them twice with the PBS containing 37 degree celsius, the media is removed and fixed with the paraformaldehyde then the slides are visualized in the microscopes. So, in the incubation assays, what you are going to do is you already prepared the lysosome in the cell, then what you do is you add the latex beads to these cells and allow them to phagocytose.

And once they are phagocytosed the phagosomes are already are been formed lysosomes are already been there because they are already been fluorescently labeled. And now what you

can do is you can just do these incubations for you know the multiple time points like you can do 5 minutes, 10 minutes and up to 1 hour. In this process, the phagosome will meet all the lysosomes and the lysosomes are going to be fused with phagosomes.

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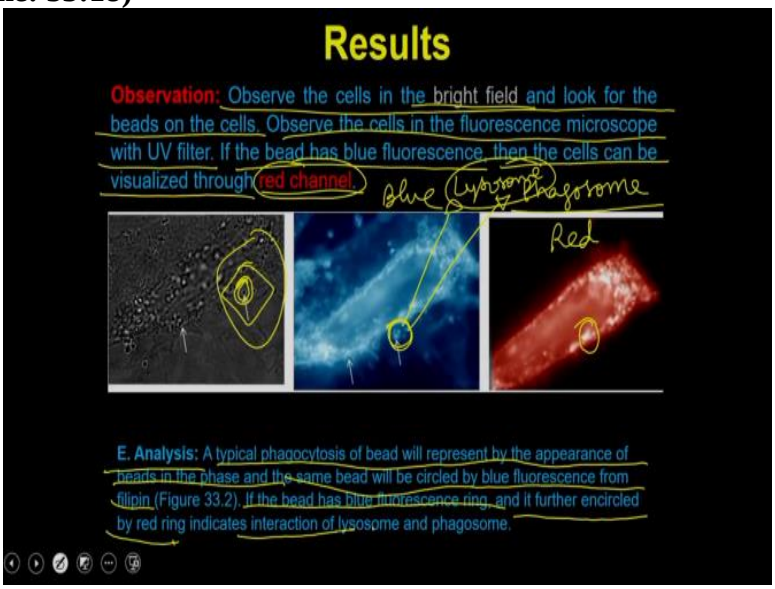

So, what will happen the when you visualize these cells under the microscope what will happen is that if you observe the cells in a bright field and look for the beads onto the cell, so what you are going to see is you just visualize the cells under the microscope, then you look for the beads under the bright fields to observe the cells in a fluorescent microscope with a UV filter.

If the bead has a blue fluorescence, like in this case, this is the bead and it has a blue fluorescence around it which means this is actually a phagosome which has been formed already. Now, once you saw the phagosomes then what you can do is then the cells can be visualized through a red channel. So what you are going to do is first you select the bead what you want to visualize under the bright fields, then you go with the blue channel.

So, in the blue channel, it is actually going to give you a blue fluorescence around it and then you go into the red channel. So, once you go into the red channel, what you will see is that this blue colored signal is also having a red color signal, which means, if you have the double signal like if you have the blue color ring, and then if you have a red color ring around it, which means these are the phagosomes, which are actually having the lysosomes as well, which means here the phagosomes is been interacting with the lysosomes.

And that is how the phagosomes has received the content from the lysosomes. So, that is how you can be able to conclude that the phagosome is been now matured, and it has formed the phagolysosomes how you are going to analyze this a typical phagocytosis of bead will represent by the appearance of the bead in the phase data which means this one and the same bead will be circled by a blue fluorescence from the filipins.

If the bead has also blue fluorescence ring and it further encircled by a red color ring indicate that the interaction lysosomes and phagosome is happening you might see that some of the beads like for example, this bead is also been formed. And this bead is does not have any phagosomes. For example, in this case, you see this is the bead, which is actually been internalized.

And you have a blue color florescence but for this particular bead, you do not have any signal into the red fluorescence which means this bead is only forming the phagosomes. But these phagosomes are not interacting with the lysosomes. So that is how you can be able to study the interaction of the phagosome with the lysosomes. And you what you have done is you have simply allowed the cells loaded with the lysosomal markers or the fluid markers.

So, that the lysosomes are being labeled with a red color fluorescent and then you allow the cell to phagocytose some material and once it will phagocytose it will going to form the phagosomes and then it is actually going to interact with the lysosomes and wherever the phagosome is going to interact with the lysosomes you are going to see a bead which is circled by a blue color ring and blue color ring is further encircled by red coloring.

So, if you have 2 rings, red and blue, so that is a place where the phagosome is being interacted with the lysosomes. So, with this, we would like to conclude our lecture here and in this lecture, we have discussed the different experiments what you can be able to perform with the help of the microscopy and I hope you might be able to design few more experiments after looking at the potential of these particular techniques and it may help you in designing the new experiment for your own work. So with this, I would like to conclude my lecture here. Thank you.