Oral Biology
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Lecture - 09
Stem cell isolation

Hi, I am Doctor Raghunandhakumar working as an associate Professor and my research background is like cancer stem cells. And we are focusing on research deals with the all cancer stem cells and also cancer cells. We are expanding primary culture cells from the oral cavity like gingival fibroblasts, gingival tissues and periodontal ligament tissues and also dental pulpal tissue.

Our main core is like we have to expand the primary cells from the oral cavities and we have to check the biocompatibility and cytocompatibility of the dental materials.

And my research strength is like checking a new drug discovery and the molecular mechanism begins in stem cells and stem cell related research and regenerative medicine also. So, I am happy to welcome you through this video to share my knowledge and to give a demo about how to expand the primary cell culture and what is the applications we are dealing with the stem cells, welcome you all.

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So, as I said earlier like we are expanding the primary cell cultures from the dental cavity like periodontal ligament tissues and gingival fibroblasts and dental pulpal tissues. And we are the one we initiated and expand the primary culture cells from the oral cavities and we check this biocompatibility and cytocompatibility.

And say for example, dentures and implants from peak and zirconium oxide and also from titanium plates and also, we are checking the pockets, sponges for hemostasis pocket sponges to check the biocompatibilities and these are all the demo. So, we have so far routinely doing the experiment.

Before I enter the experiment, I want to show that the accessories of whatever we are using for the cell culture . So, these are the dental materials accessories we are using for to check these cytocompatibility and biocompatibility. This is the basic routine consumables and accessories whichever we are using for the cell culture and I am now going to give a live demo for cell culture technique.

So, before we enter into the live practical demo about the culturing and isolation and culturing and expanding the stem cells. I just want to give a small introduction about the in vitro cell culture and. Generally, why we are doing in vitro cell culture is like we want to check say for example, we want to check any new inventions like or any new material to check their cytocompatibility or biocompatibility before entering into in vivo culture.

So, that we are introduce these techniques and some cell experiments they were already listed and then the standard operating procedures are already existing we just following the SOPs to check the bio materials biocompatibility and check.

As you all know about our human body made up of blood, flesh and bones, but many of them are not aware about that whole body is arise from the single cells. So, when we when I started my research career, I just wondering about how does the single cell become the body human body.

So, behind on it I started my research and then we expanding like that the single cells is none other than it is a stem cells. So, these stem cells are very unique and they have a unique characteristics they can be divided or they can be differentiated to the any form of our body parts. So, our main focus is how the stem cells differentiated in different form

of our body parts or different form of tissues or cells and what is the mechanism behind on it.

So, in that being a dental domain we only focusing on the dental pulpal tissues and dental pulpal stem cells and check how it is helping for a re-generative applications especially in dental domain. Before we know about the in vitro cell culture we should know about the basic fundamental and what are the equipment's we are using for the in vitro cell culture.

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First one, is like for a in vitro cell culture the main important thing is like is CO 2 incubator, why it is very important is like our human body have maintained the same constant temperature and constant oxygen supply and constant carbon dioxide supplies it will be there for inhalation exhalation.

So, that in vitro culture ideology is like we have to mimic the human body at that atmosphere into the culture room. So, we have here that CO 2 incubator having 37 degree Celsius and 5 percent in CO 2 incubated as CO 2 supply and humidity about 92 percent.

So, and moisture content always supposed to be as like our human body conditions. So, this; so, in this culture setup unlike in the CO 2 incubator setup we could be able to mimic the our human body systems that the body temperature and its oxygen and CO 2

supplies and etcetera's. And here some cells were maintaining as for the experiment purpose and apart from that, this is the inverted fluorescent inverted microscope.

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To check the cell morphology before to monitoring whether the cells are healthy or unhealthy or whether it got contaminated or not contaminated. For that this is the monitoring purpose to track how our body systems are monitoring with the help of in that normal ECG or what that, likewise the cells also monitoring with a morphological study.

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Apart from that, and this one is inverted fluorescence microscope from these we could be able to see the live cells with the help of fluorescent dyes like Alexa Fluor, Dapi and Cy 3. So, usually that fluorescent microscope has three different color range, one is red and green and blue. With the help of these different dyes we could be able to track the particular cells morphology and their internal organs organelles and the nucleus also ok.

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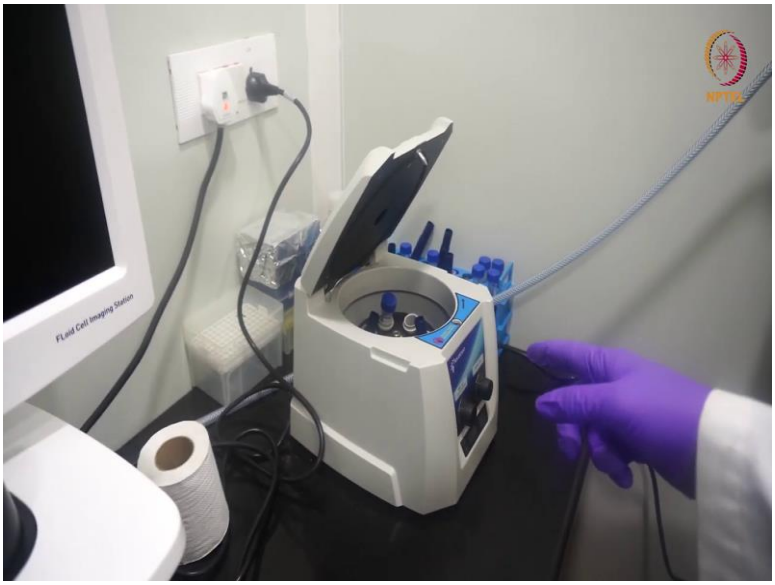

And centrifuge is also very important thing to separate and isolate and culturing and further subculturing the cell lines ok.

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So, this is the working platform this is the in vitro cell culture working platform so, called biosafety cabinet. Our biosafety cabinet is class 2 b 2. So, here the mechanism means that the outside air should not enter inside and inside air should be circulated and then it will go towards the outlet that.

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The purpose of this is to avoid the contamination by blocking the outside air should not enter into inside, ok.

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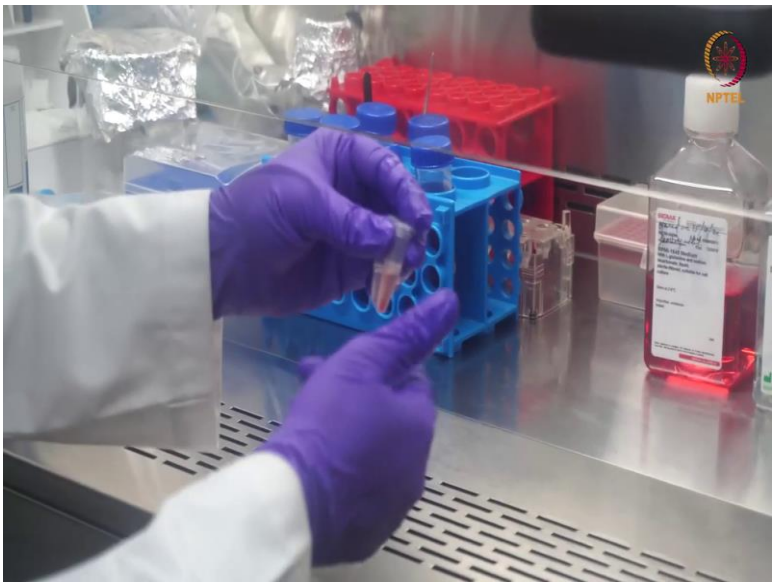

So, now I am going to give the demo about how to like isolate these gingival fibroblasts and the gingival tissues. Usually, we collect the samples from the volunteers patient's after the clearance of ethical and no objection from the patients.

So, we collect the gingival tissues and usually there are two different type of isolation is there; one is, chemical enzymatic digestion, another one is mechanical digestion. It is nothing but that the mechanical digestion is nothing but there is a chopping of small pieces of each tissues with the sharpened blades.

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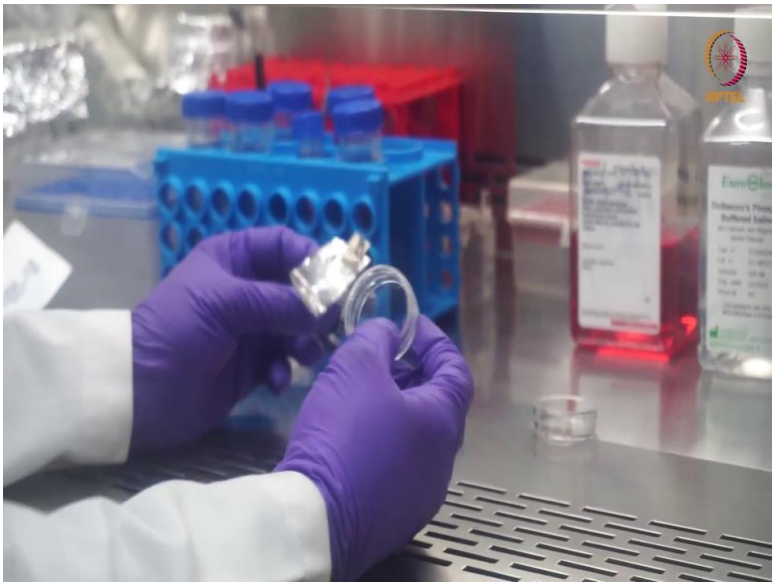

Yes, and that enzymatic digestion is nothing but chopping the tissues after that we have to incubate with the collagenase type 4 to for 30 minutes to dissociation of tissues as a single cells. And these enzymes will help to dissociate the cells become single cells from the tissues.

So, now I am going to give step by step demo, how we are digesting the tissues with the help of sharpened blade forceps and sharpened blades and also for expanding the isolating and expanding the gingival fibroblast we need major one is sharpened blade and this one is petri dish, Canon dishes for chopping and then isolating the gingival tissues for culture expansion.

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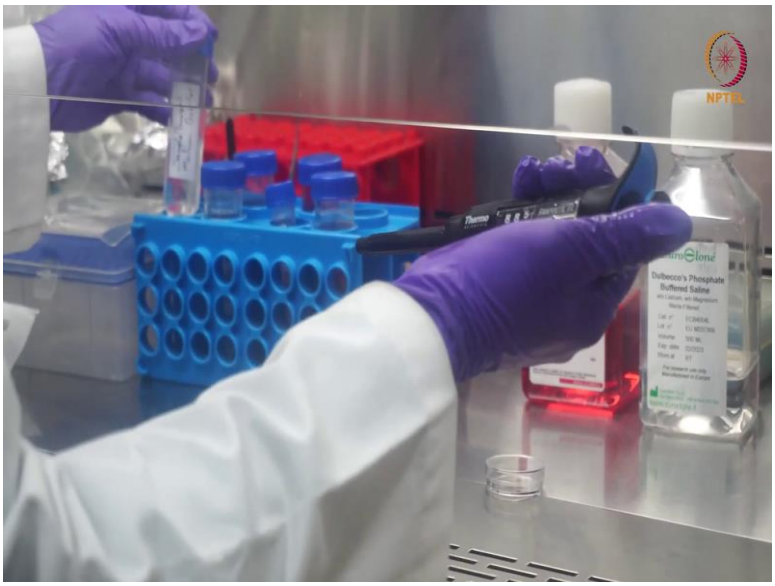

This is micro viper to helping it is help to collect the media or any solutions from the stock to our desired place, ok.

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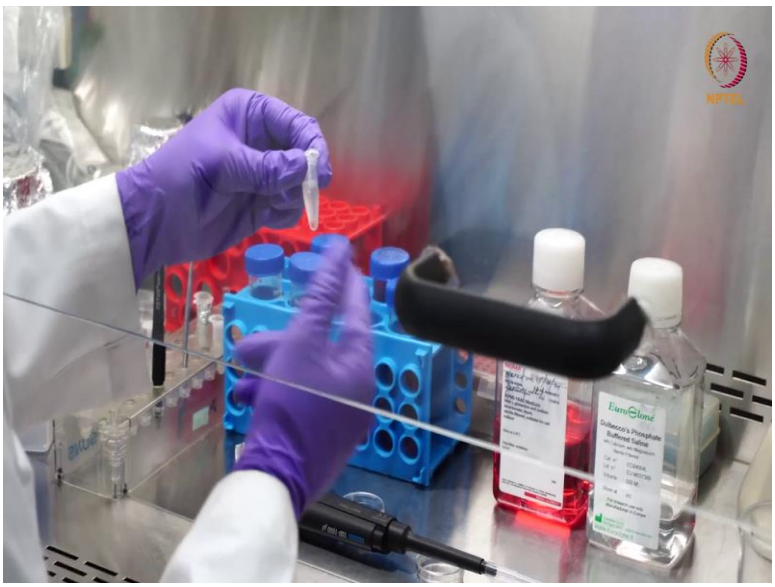

So, before we start to expand digestive tissue. So, we should wash with your one wash with the antibiotic containing normal saline, ok normal saline and make sure that tissue is supposed to be perfused with the normal saline and try to avoid as much as that blood strain or whatever the debris supposed to be discharge are separate from the tissues, before we start the mechanical digestion of the tissues.

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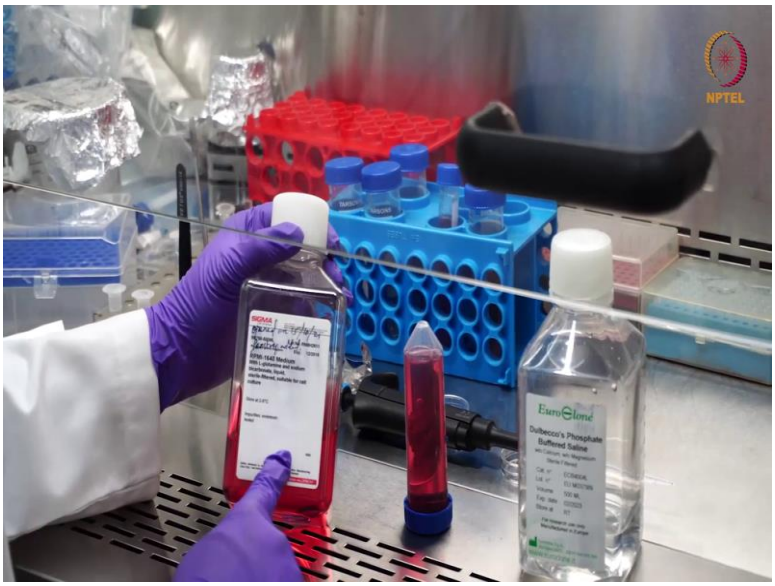

So, before we start the procedure I would like to show the what are the chemicals and solutions we are using for the cell culture. So, generally for the cell culture we have to use the cell culture media which contains all micro nutrients see you know like there are gal glutamate sodium bicarbonate liquids, sterile water and suitable for like it supposed to be it contains all micro nutrients and endotoxin free and pathogen free this one.

And we purchased from the sigma and apart from that we have we are using phosphate buffer saline without calcium and magnesium, why we are using without calcium and magnesium mean it helps to detach the cells from the surface bottom usually. And cell culture all items whatever we are using here the media or any antibiotic solutions or phosphate buffer cell all supposed to be cell culture grade, and.

Whatever we are dealing inside the culture room it is not a matter our primary purpose of that doing all the procedures inside the (Refer Time: 13:04) to try to avoid the contamination and like experience can help you to improve your skill of handling the culture cells and to avoid as much as contamination free cell cultures. And we are the primary source to for the contaminate cells.

Because that what are the materials and the cells all always inside the four walls, but the we are the one accessing outside and then coming inside. So, while entering inside and going outside we supposed to be sterile with the help of IP 70 percent alcohol it helps to sterile.

We are supposed to be in the sterile condition while accessing or dealing all the tissues and cells inside the culture room. And we suppose should not touch any outside and so, here we collecting the gingival tissues from the human being and now, I am going to digest with the mechanical method like chopping with the sharpen blade.

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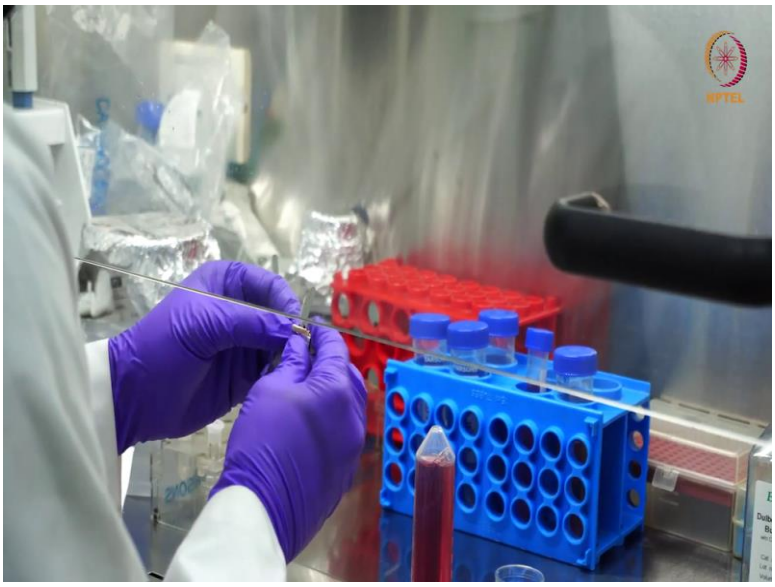

So, already I have made one wash with normal saline contains antibiotic solutions. That, antibiotic solutions is nothing it is a penicillin and septomixine after a one wash gentle wash.

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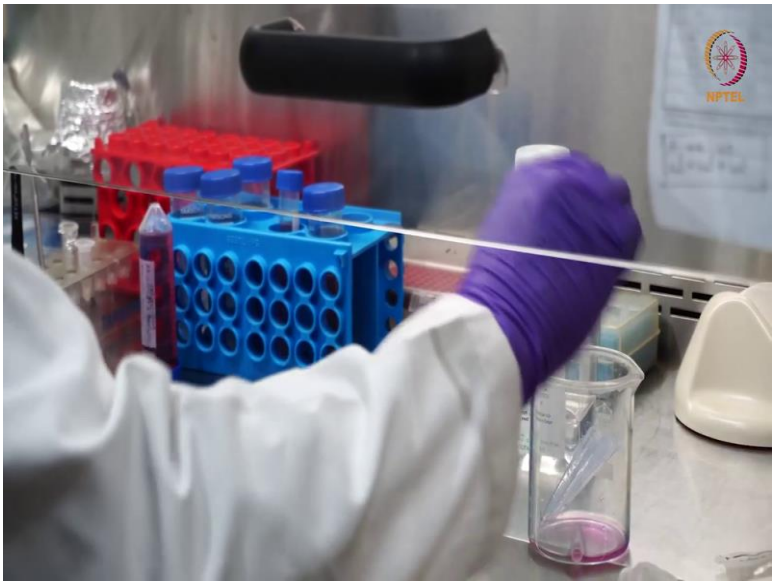

We discard the antibiotic containing saline solutions. Then, we wash with media, which we are going to maintain it as tissues after the digestion.

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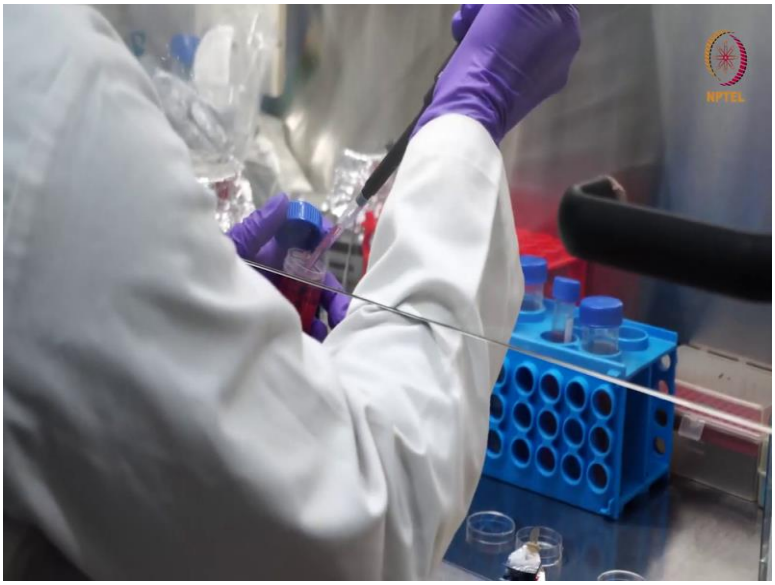

After washing with the media that we are going to discard the media without any disturbance of the issues. Then, it moves with the media for a while until we take off the tissues from the tubes for the enzyme and mechanical digestion.

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In middle of the experiment frequently you wipe with the 70 percent alcohol to avoid the contamination. So, now, I washed the tissues with the saline contained antibiotic solution

afterwards I transfer the tissues with the micronutrient enriched media. Now, the cells or tissues are ready for the mechanical digestion.

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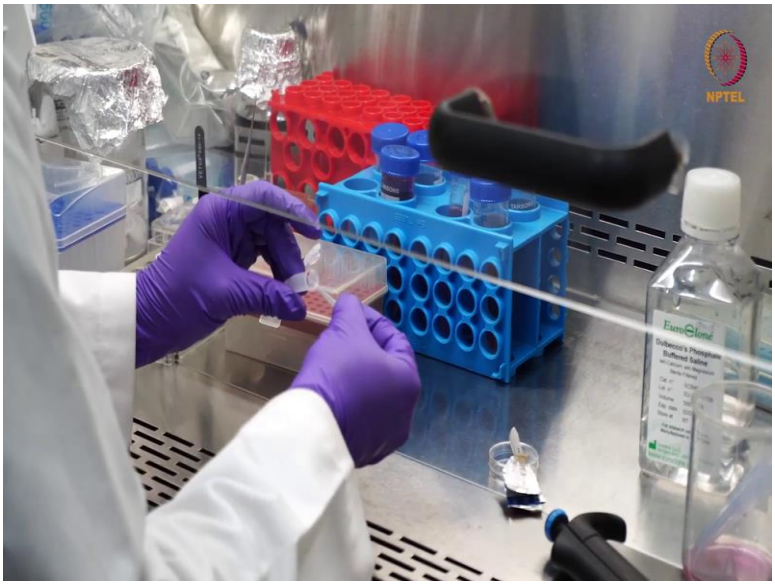

So, now I am going to gently transfer the tissue human gingival tissues into petri dish. Make sure that the tissues suppose should not be air dry. So, now, I am going to add few drops of the media let it be immersed into the media these tissues. As I said earlier that, there are two type of digestion one is mechanical, another one is enzymatic.

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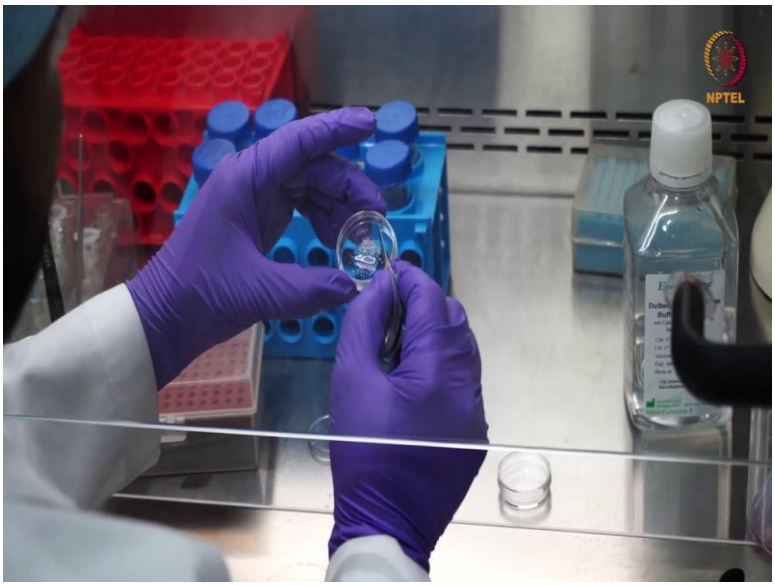

Now, I am doing mechanical digestions it is nothing but like a pinch of tissue has chopped with the sharpened blade with the fine pieces. So, while doing the small pieces. So, some tissues dissociate become a single cell sometimes some bunch of tissues bunch of cells become a colony try to chop it as much as fine pieces. See here initially it was a pinch of tissues, now it has become fine pieces with the help of sharpened blade I chopped it in a fine pieces.

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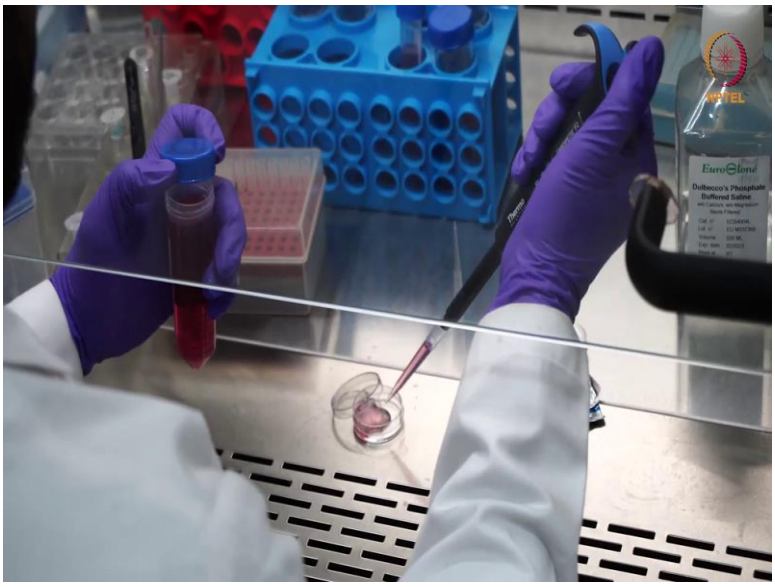

So, now we are half of the way in the experiment middle and now, I am going to add micronutrient enriched media, which contains glutamate many essential amino acids and salts basic salts. It will help to retain the cells become live and these media also contain 20 percent fetal bovine serum. So, now, that fine pieces has immersed in the micronutrient enriched media.

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So, I immersed with the micronutrient enriched media with our interest of tissues from collected from the gingival fibroblasts. And now, I am going to cut and expand the tissues in a single cell isolation and expanding human gingival fibroblasts with the enzymatic digestions. So, enzymatic digestion is nothing but incubate the with the fine chopped tissues with collagenase type 4 this will help to dissociate the cells from the tissues as a single cells.

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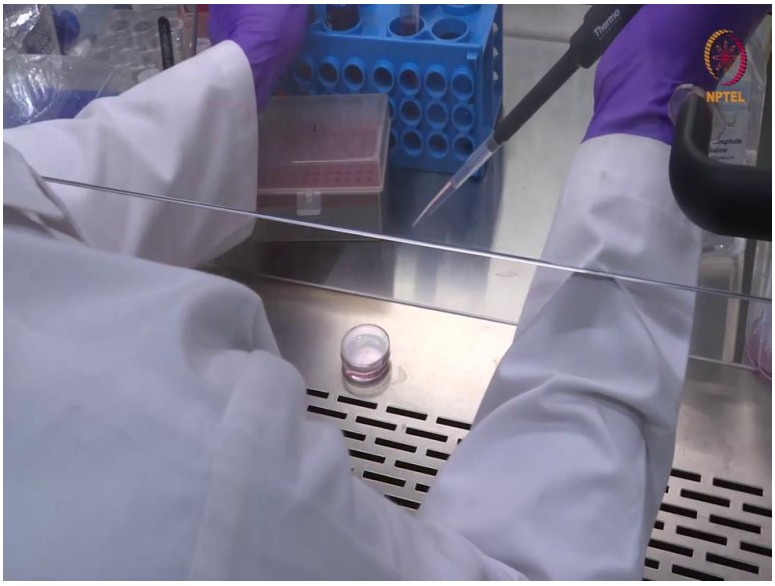

After incubating, now, I am starting the preliminary step of the enzymatic digestions. There is only slight changes will be there in enzymatic digestion when compared with the mechanical digestion. What we are doing is like for mechanical digestions just we chop into small pieces, but in enzymatic digestion after small pieces we incubate the fine tissue pieces of these tissues with the enzyme collagenase type 4, which will help to dissociate the single cells from the tissues.

So, now I am making it small pieces. As fine pieces with the help of the sharpened blade surgical blade we incubate the cells with collagenase type 4 enzyme for 30 minutes. Try to collect the left-over small pieces stick the side edges of the sharpened blade, which helps to yield more single cells from the tissues.

So, now I am adding collagenase mixed condition media for enzymatic digestion, which helps to dissociate the cells from the tissues. So, why we want to do this such a dissociation means, the duration of single cell formation from the tissues that they will be reduced when compared to mechanical digestion; that is why we suggest if the sample and the study is very precious go for enzymatic digestion rather than to fastening the experiment to accomplish.

So, after 30 minutes we replace the media with the micronutrient enriched human gingival fibroblast condition media. So, this is the preliminary step of how to culture the primary cells from the tissues, in any source; like human, animal model. Say for example, bone marrow from the mice to isolate the MSC cells Mesenchymal Stem Cells from the bone marrow, ok.

So, now I am going to put inside the incubator and very next we should monitor the dish. After the enzymatic digestion I am going to place the tissue containing petri dish into the CO 2 incubator and very next day we have to monitor whether the media has discolorate or any contamination is there.

If is there any no contamination or if there is no de-coloration we supposed to leave it without any disturbance for minimum 3 to 4 days and let it to attach the tissues in the bottom of the petri dish. For the attachment the cells will start arise a single cell from the tissues and it will take minimum 1 week to 10 days ok.

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Stop.

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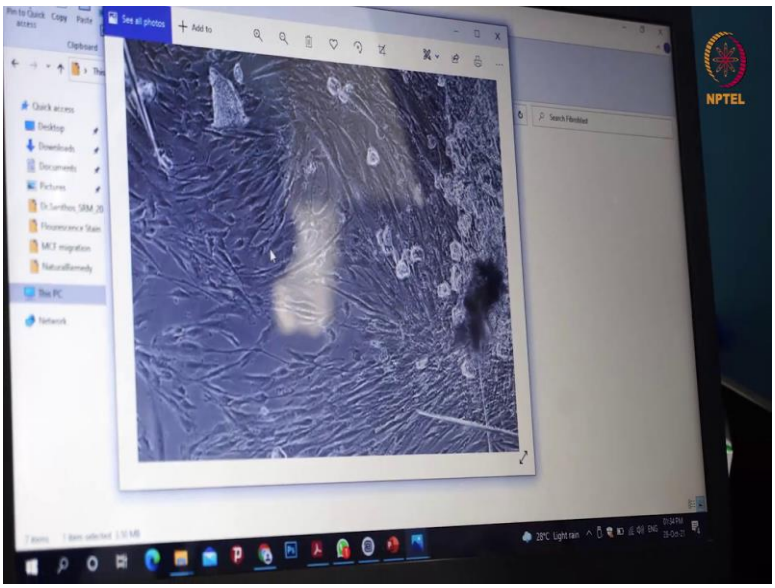

So, after cell has attached tissue has attached at the bottom after 10 days. The cells will start to arise as single cells from the tissues and then from that inner core is nothing but the fine chopped small pieces of tissues arise. And you could be able to see the

surrounding places there is a single cell has aroused from the tissues this is called like after 10 days, we could be able to see the single cell formation aroused from the tissues, ok.

So, after some days that cells will gets wide and spread and you could be able to see monolayer cells aroused from the tissues and it start to spread all over the surface area of the empty space of the petri dish. So, we leave it the cells let it be, until it gets to start to spread all over the space to cover the empty space.

After that monolayer cells formation we trypsinize the cells with the help of trypsin it is a enzyme it will help to detach the cells from the surface bottom for the further passages. Usually, that primary cultures and when it reaches the p 2, then only you will get the monolayers that mono homogeneity.

Usually, the monolayer cell culture that homogeneity will start from the p 2 passage before that there are bunch of different type of cells will be there like fibroblast, epithelial cells and also some RBCs also will be there. But after a week that second passage you will get the homogeneity cells will be there, uniform morphology and same cell from the majority of population cells will be in 90 percent of the cells will be fibroblast.

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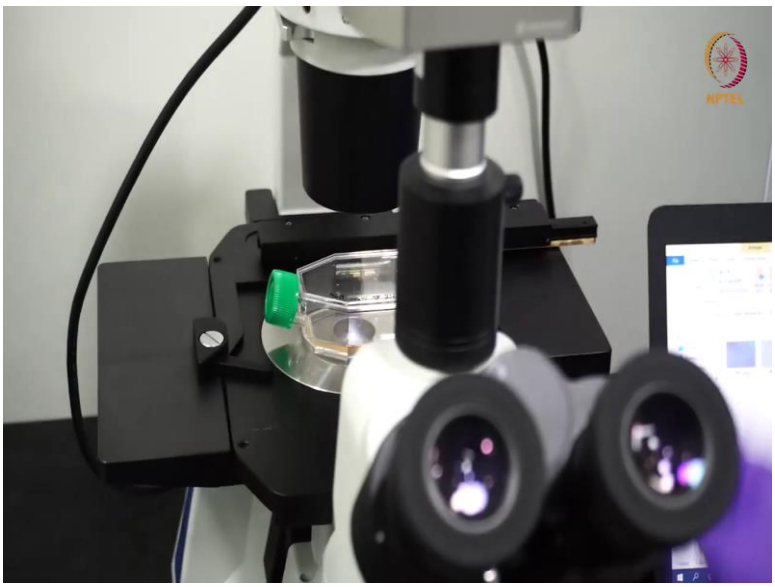

Now, I am going to give the small example for homogeneity of fibroblast cell. Here we taken up into p 2 passage sorry p 2.

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This is the ideal example for the human fibroblast with the uniform morphology. You could be able to see all the cells are there in same morphology and also spindle shaped morphology and same sizes its so, called homogeneity

So, here only cells are there; there is no contamination, there is no debris, there is no any bacterial contamination. If it is any bacteria means you could see this is the live imaging you could be able to see any movement mobility is there; there is no motility only the cells are there. We are screening the live cells with the help of live cells with the help of inverted micro phase contrast microscope.

Whatever I showed you phase contrast normal morphology microscope. Now, I am going to do another one as in it is live cell imaging with the help of acridine orange, which emit green fluorescent. So, green fluorescent means like whichever healthy and live cells will obtain or uptake the green dye with, if and also under the microscopy while its absorbing it will emit the green fluorescence. So, it confirming that the cells are live there is no any cell death.

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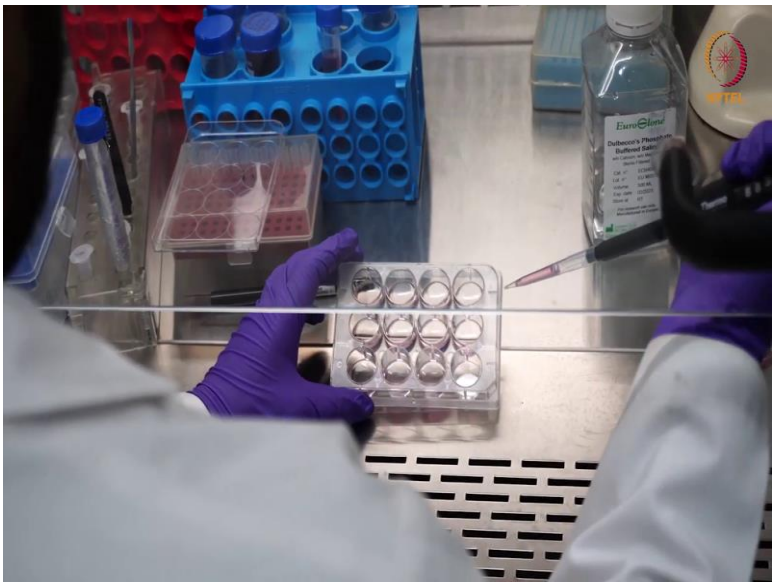

Now, what I am doing is like to check the cell viability with the help of acridine orange staining. So, I am replacing media already I pre seeded the cells last day itself into the 12 m micro plate.

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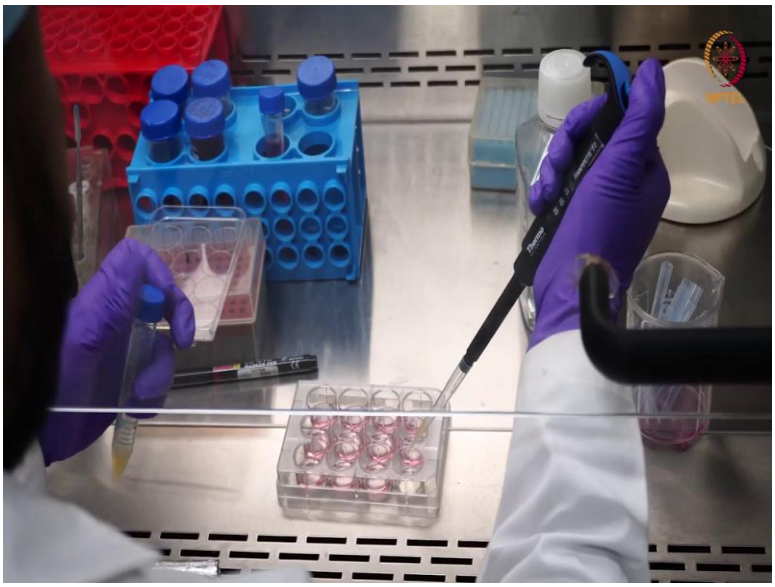

Now, I am going to add fluorescent dyes that acridine orange onto the cells and incubate for few minutes. So, after the incubation for 4 minutes I am taking off the dye and gently replace with phosphate buffer saline.

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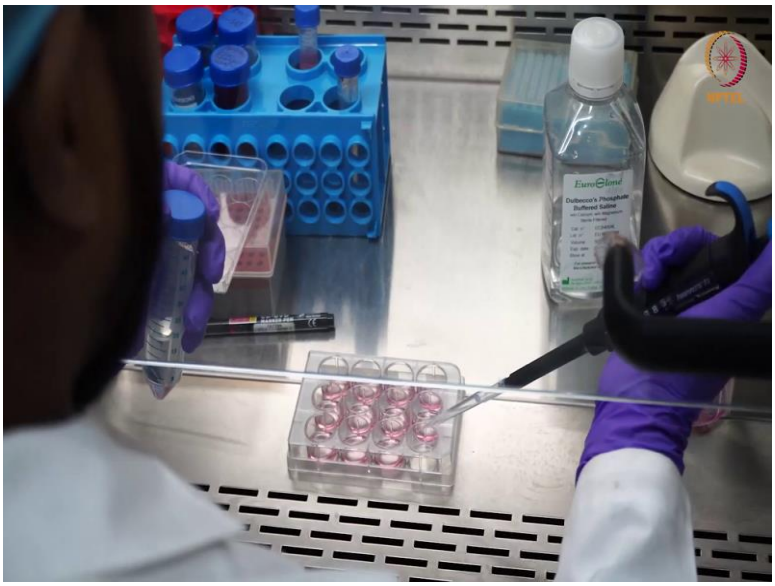

So, again one more wash to discard the leftover dye and with the help of phosphate buffer saline, after discard the fluorescent dye. Now, I am going to place it in the inverted fluorescent microscope to absorb the cells and whether it has a pick the dye or not and with the help of fluorescent microscope.

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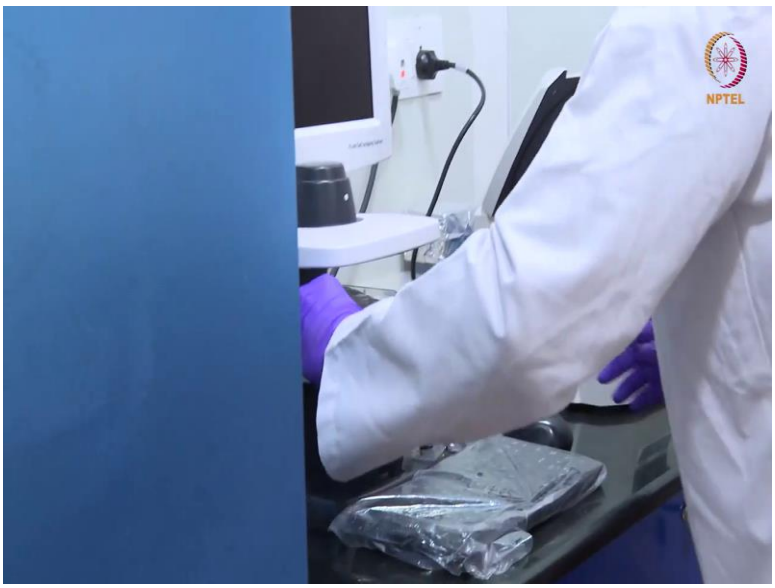

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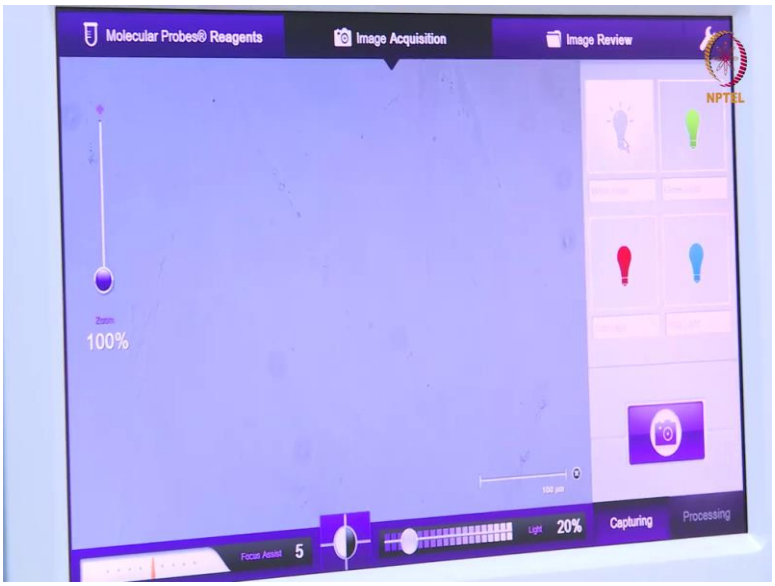

So, this is phase contrast bright field you could able to see the cells here,. See here these are cells and you could able to see here there is a nucleus there is a two dots its nucleolus. It is a bright field, now we already added a green fluorescent dye so, called acridine orange dye.

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Now, I am going to switch into green fluorescent plan. So, see here the cells uptake the green fluorescent dye and emitting the green fluorescent while absorbing under the green field. So, usually that acridine orange, which is the categories of when the cells are

emitting green color its confirming that the all cells are healthy live cells, when it turn become yellow or red means its earlier apoptotic or late apoptotic it turns red color means it become necrotic cells, but all field showing green color.

So, we are confirming that all cells are healthy and normal,. Yes, see here usually primary culture cells are isolated from the dental pulpal tissue this one, this cells; and we add green fluorescent dye and absorbed under the inverted phase contrast from fluorescent microscope. So, in green under the green fluorescent lamp it emitting in a green color that cells.

From this session, I shared my knowledge and talking skill about the how cells isolate from the tissue human tissue. Primary cells isolate from the human tissue for the further experiments and also for regenerative applications also translation research. Our lab also dealing with some bio stuff hold biomaterial related studies also we are doing it. And this is our lab normal routine experiments and then research so far, we are doing here.

Thank you.