Tissue Engineering
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Lecture - 18
Cell Isolation - Part 2

In the previous class, we were talking about cell culture, and we were looking at different aspects of cell culture, which are required for Tissue Engineering. And we talked about isolation techniques; we were talking about different ways cells can be isolated based on different things. We spoke about cellular characteristics such as surface charge, and adhesion can be used for separation and isolation of cells.

We will move on to the other aspects. We will talk about how the size and density of cells can be used. And we can also talk about cell morphology and physiology, surface markers and antigens, and so on, which can be used for the separation of cells.

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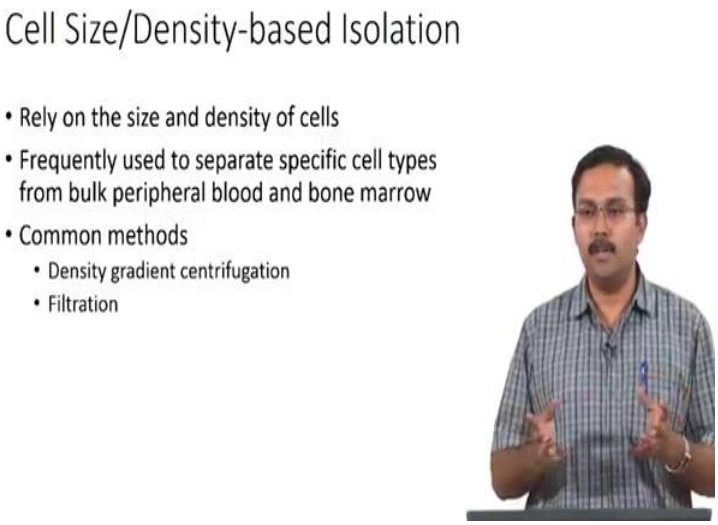

We will first talk about that size and density-based isolation. This is actually a crude way of separating, but it is quite effective for certain types of cells, and it is being done extensively. This relies on the size of the cell and the density of the cell. And techniques like simple centrifugation or filtration are used to make sure that the cells are separated.

This is frequently used to separate specific cell types from peripheral blood or bone marrow. When you have this kind of liquid source, you can just spin it down, and you would end up with the pellets in the bottom, or there will be different layers. And you can get different cell sources in the different layers, so that is what this is used for. This is very commonly done even in clinical settings and so on.

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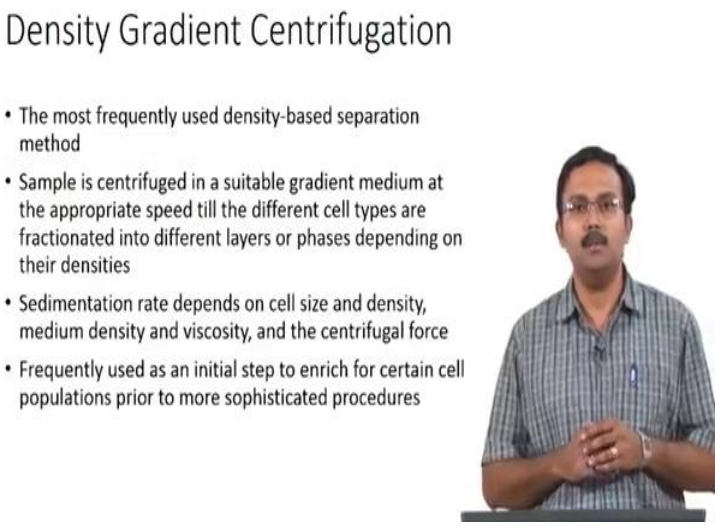

Density gradient centrifugation is the most frequently used density-based separation method. Here, the sample is centrifuged using a suitable gradient medium at an appropriate speed till the different cell types are fractionated into the different layers or phases depending on their densities. This medium will contain things of different density, which will all align itself, and the cells will also align themselves with that particular density. The sedimentation rate of the cells will depend on the cell size and density, the medium density, and viscosity, and also the centrifugal force, which is used.

With respect to centrifugation processes, the speed which is used will have a significant role in how the cells are isolated. Because you will experience a lot of shear stress when you are having this high centrifugation speeds. That is one of the reasons people use this for separating cells in peripheral blood and so on, which can withstand the shear rates.

If you have a cell population, which cannot withstand the shear rates, then you would not want to go something like centrifugation. This is usually used as the initial step to enrich certain cell populations before some more sophisticated techniques like FACS and so on.

You would not want to use this as the only thing to separate unless you are just trying to isolate platelets and so on.

If you take blood and you perform centrifugation, you can easily get platelets, like platelet-rich plasma can be easily obtained. But it does not mean that it will be only the platelets you have got. Let us say if you want something very specific; if you want only one specific type of cell, then it becomes more crucial to have something more sophisticated down the line.

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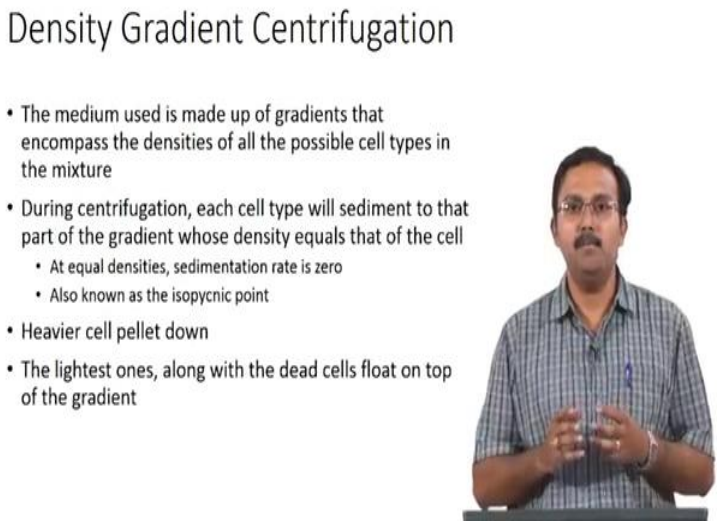

The medium which is used is made up of the gradients that encompass the densities of all the possible cell types in the mixture which you are using. During the centrifugation process, each cell type will start sedimenting, but it will not go down to the bottom. It will reach some kind of equilibrium at the gradient, where the density is the same as the density of the cell. This, you would have even seen with multiple layers of different liquids. So, it is not everything is going to go to the bottom.

At equal densities, the sedimentation rate will be 0, and at this point, cells will settle, and that is where it will not move further, even if you continue with the centrifugation process. This point is called the isopycnic point, and you try to do this to ensure that the cells are separated.

If the cells are much denser than anything in the medium, they will start to form pellets at the bottom. And these pellets can be removed very easily. People will take out the pellets and throw it away if that is not needed and or use it for further studies. The lightest ones and the dead cells will float on the top of the gradient. So, you would have some amount of cell damage in this kind of technique; you are going to have some shear, and there is going to be some damage to cells. So, the dead cells which are lysed will we will very light, and they will end up floating on the surface.

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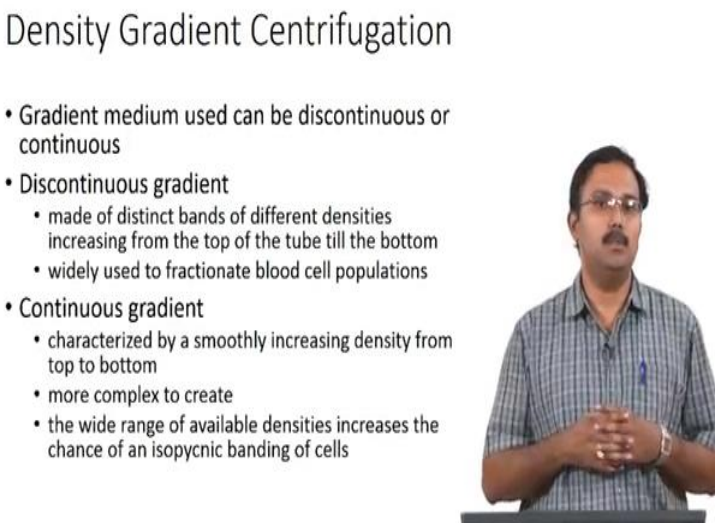

You can use two different types of gradient medium; the gradient medium could either be discontinuous or continuous. The discontinuous medium has basically distinct bands that can actually be obtained. This is made of distinct bands with different densities. And what happens is, you would have these densities, which will help in fractionating the blood cell population. In the continuous gradient, then you are going to have a smoothly increasing density, wherefrom the top, you would have lighter density, and the bottom would be the heaviest thing.

But it is not going to be clear regions; you are going to have a smooth gradient. And this kind of gradient medium is more complex to create. A wide range of density is available when you use this, which means the cells can have a better chance of isopycnic banding. When you are using a discontinuous gradient, you need to have enough number of bands for the cells to reach the isopycnic point, otherwise, the cells are going to float

somewhere in between, and you are not going to isolate them effectively. So continuous gradient is better for isolation, but it is more complex to create.

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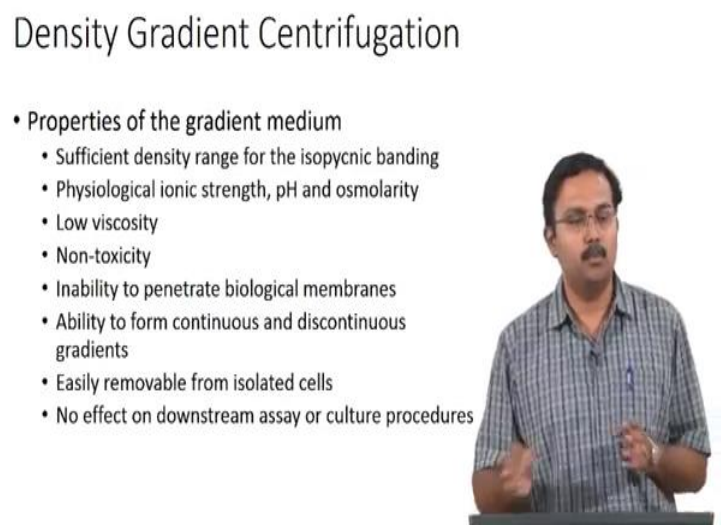

What should be the property of a gradient medium? A gradient medium should, first of all, have a sufficient density range for the cells which you are using, so that isopycnic banding will happen. If you are going to have cells of different densities, all the range should be taken care off; otherwise, the cells might just pellet out, or it might just start floating on the top, or it will get mixed up in the interface regions which is not a good thing.

The gradient medium should also have pH and osmolarity and ionic strengths similar to the physiological condition. So, why are these important? What can happen if say, for example, osmolarity is different?

Student: Cells will not maintain integrity.

Yeah. The cells can actually lyse. Cells can either shrink or swell and basically kill them. The gradient medium should ideally have low viscosity because once it becomes highly viscous, it becomes very difficult for the cells to move around. And you have to give higher shear for it to separate, which will cause damage to cells. This medium should also be non-toxic; it cannot kill the cells which you are trying to separate. It should not be able to penetrate the biological membranes because, if it starts going in, then it is

going to damage the cells, and like maintaining the osmolarity, it can help in that aspect. So, it should be able to form continuous and discontinuous gradients, depending on what you are trying to do.

And, it should also be easily removable from the isolated cells because whatever cells you are isolating, then should not have a problem of actually separating it from the gradient medium. It should not just get adhere to the gradient medium or should not become too complex to separate them. So, there should not be any effect on downstream assay or culture procedure which are going to be done in the future.

Whatever cells you are isolating, it should not just for fun that we are doing that. So, these cells are going to be used for something. If you are going to be using it for something, then whatever procedure you are using for isolation cannot affect the cellular properties for the downstream processes. So, the application should not get affected because of the gradient medium. We need to make sure that those aspects are also taken care of.

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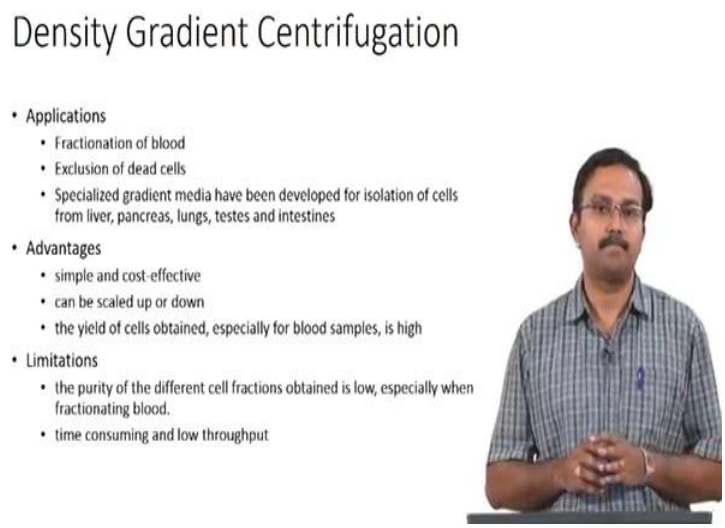

The most common application for density gradient centrifugation would be fractionation of blood or exclusion of dead cells. So, if you just want to have only the live cells and then you can easily take out the dead cells on the top. A specialized gradient media can be prepared for isolation of cells like from liver, pancreas, lungs, testes, and intestines; this is not common gradient medium that is used for blood.

Student: It is not used for blood; there is no gradient medium, which is used for blood?

No, gradient medium can be used for blood as well, but without gradient medium also you can have a density-based separation in blood. So, you just take blood, and you spin it, and you are going to get different layers. Has anybody done those experiments? We do not have a clinical biochemistry kind of lab, but if we did, you would have done that. Clearances are usually a pain to get; the ethical clearances for just drawing blood would be a problem. That is why we do not do these experiments. But you guys should do this a part of biofest or something.

There, people would not ask you for ethical clearances. You can just draw blood from somebody and show some of these experiments. The advantage of this technique is it's very simple and cost-effective. It can be scaled up or down; you can have 50 ml centrifuges, where you do it, or you can have 1.5 ml centrifuges where you do this fractionation, and it is reasonably scalable. And the yield of cells obtained, especially for blood samples, is quite high; it is not like you are going to lose the cells during this process, so the yield is reasonably high. However, the limitation is the purity of different cell fractions is not very good, and it is usually low; it can be time-consuming and low throughput because each sample can be processed individually only. You cannot process multiple samples simultaneously; it is quite challenging. You can have motors with multiple samples, but you still will be able to do only whatever the router can deal with. So, if you are going to use 15 ml falcon tubes, you can only load like 8. So, you can only do that many together, or you need multiple centrifuges to work with.

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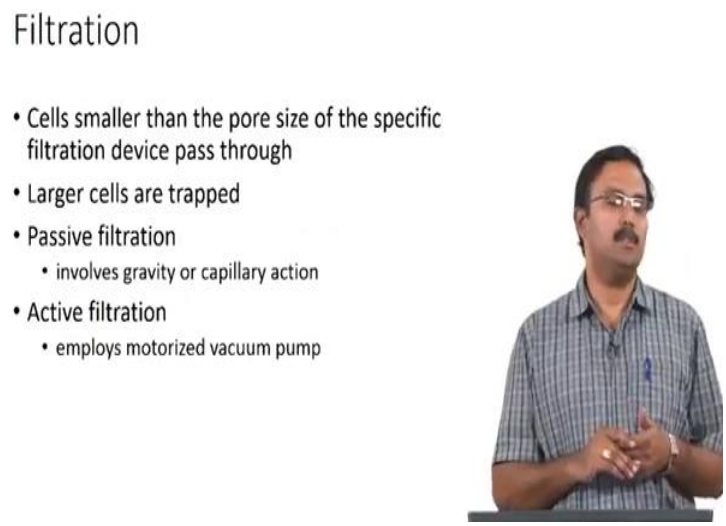

Filtration is another simple process where cells are separated based on size. Cells that are smaller than the pore size of a filtration device will pass through, and the larger cells are trapped. There can be two ways of filtration; one is a passive filtration, and the other is an active filtration. A passive filtration is a process where only gravity or capillary action is used for separation, and active filtration uses some kind of a motorized vacuum pump.

The applications are, this filtration is almost exclusively used in cancer biology specifically for isolation of circulating tumor cells, which are much larger than healthier cells. So, it is easy to isolate them from healthy cells. The advantages it is very simple and easy to reproduce, it is quite a high throughput because you can just keep having a continuous flow system where you can process multiple samples. And the filters which are containing the captured cells can be directly used for downstream assays because you are not changing the cell in any way, you are not adding any media or anything to the cells so the cell property would not have changed. You take the cells, and you can process it for any downstream application without any issues.

But the limitation is there are very poor specificity and purity. Any cell which is large is going to get entrapped in that. So, if you have a macrophage that is very large, it will come along with tumor cells; you cannot prevent that. Surface phenotypes can be lost, especially if you are going to use something like an active filtration process, where you have a motorized vacuum pump that is being used.

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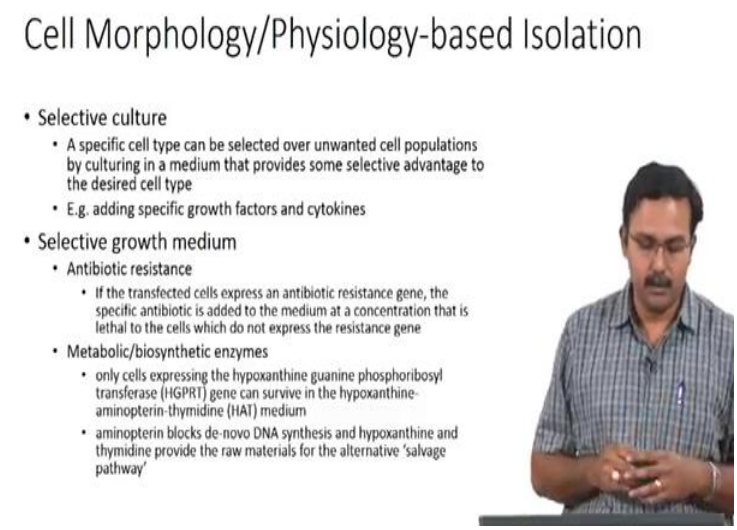

Other than density and size, morphology and physiology of the cell can also be used for isolation of the cells. What people do is selective culture, where a specific cell type can be selected over an unwanted population, like culturing it in a medium that will provide an advantage to the type of cell which we are looking at.

This could be done by adding specific growth factors or cytokines, which will help that particular cell grow while other cells do not use those nutrients in the media. What you do is, you create a selective growth medium, which could either be an antibiotic resistance medium, which is usually used for isolation of engineered cells. So, you could have cells, which are transfected and have an antibiotic resistance gene, and then you can use that antibiotic; every other cell will die, and only the cells with that antibiotic resistance will survive. This is a very common technique used in molecular biology to ensure that you take out only the transfected cells, and then you can use that for whatever studies you are going to be working with.

Metabolic or biosynthetic enzymes can also be used if there are cells that specifically express certain enzymes, then you can create media, which can be used only by the cells which produce these enzymes. If the cells are not producing the enzyme, they will not be able to use the media, and they will just die out. One common example of that is the hypoxanthine guanine phosphoribosyl transferase.

Student: HGPRT.

HGPRT. So, this is a gene that actually can express certain types of enzymes, and this can take up the HAT medium. If the cells do not produce this gene, they cannot take up the HAT medium, and they will just die.

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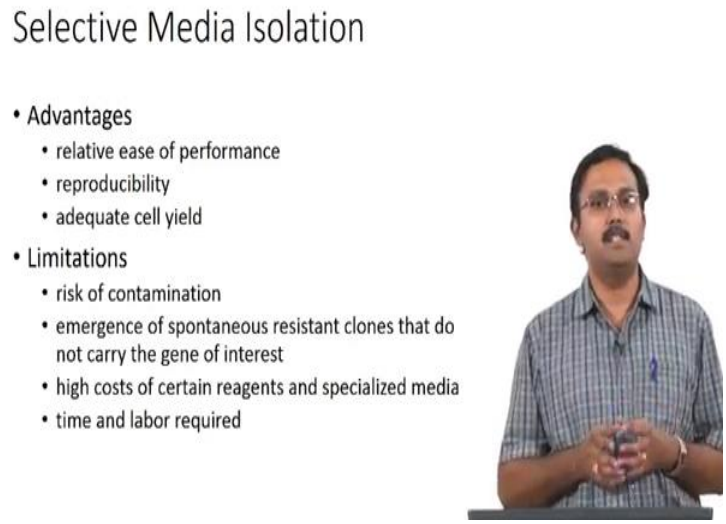

The advantage of this selective media isolation is it is again reasonably simple. All you are doing is culturing the cell; you are just using different media. And it is quite reproducible because you are looking at the property of the cell, which is unique to the cell type which you are going to be working with. It will give adequate cell yield because there can still be transfected cells or cells with the desired types which can die, and you will not be able to recover them.

The limitation is there is going to be a risk of contamination because you are looking at the culturing process, which is usually a much longer process than centrifugation or a filtration process. Cell culture is going to take one day or three days or seven days, depending upon a type of the cell you are working with. There can also be an emergence of spontaneous resistant clones that do not carry the gene of interest. So, you can always be isolating some of the cells, which are not transfected. They just have some mutation, which creates the resistance which you are using for screening.

There is also a high cost associated with this process because any time you work with cell culture, it is going to be very expensive. Now, it is also time-consuming, and a significant amount of labor is required.

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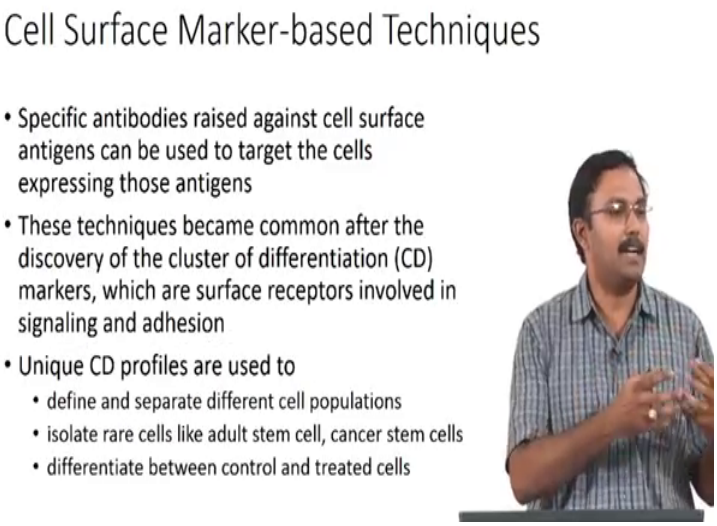

Another factor that is used for the separation of cells is cell surface markers. These are used for very specific separation. Antibodies are raised against cell surface antigens, and they are used to target the cells which are expressing those antigens. So, these techniques became common after the discovery of the CD markers; so, the CD is a cluster of differentiation markers. These are surface receptors that are involved in signaling and adhesion. When this was identified, people realized there are unique markers on cells that can be used for the isolation of cells. These unique CD profiles can be used for defining and separating different cell populations. So, people use CD markers to identify cell populations, as well.

After isolation, also even if you have to confirm that this is a particular cell type, then CD markers are very commonly used. These can be used to isolate rare cells because that particular cell type might have a combination of CD markers, which can easily be used for specifically targeting them and isolating them. This can also differentiate between control and treated cells.

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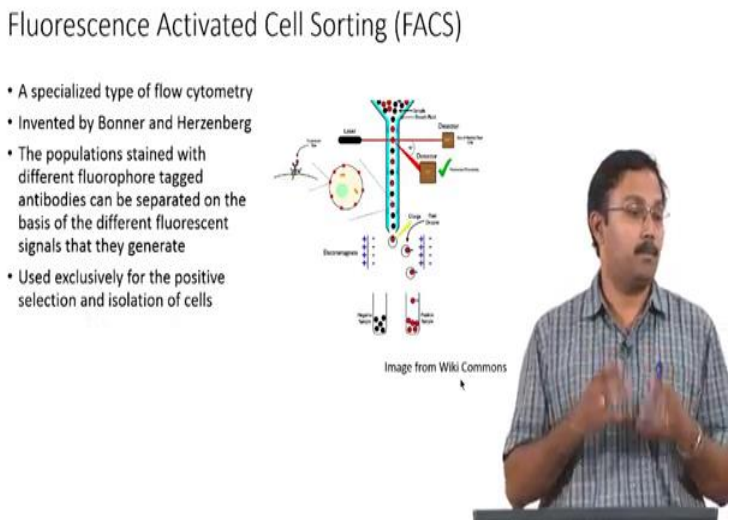

One process, where this kind of surface markers are used for separation is fluorescence-activated cell sorting or the FACS. This is a specialized type of flow cytometry. This was invented by Bonner and Herzenberg. And the populations are stained with different fluorophores tagged antibodies, which can then be separated based on the fluorescent signals which are generated by these cells. These are used exclusively for positive selection and isolation of cells. We talked about the positive selection and negative selection, and so on earlier. So, this is used for positive selection.

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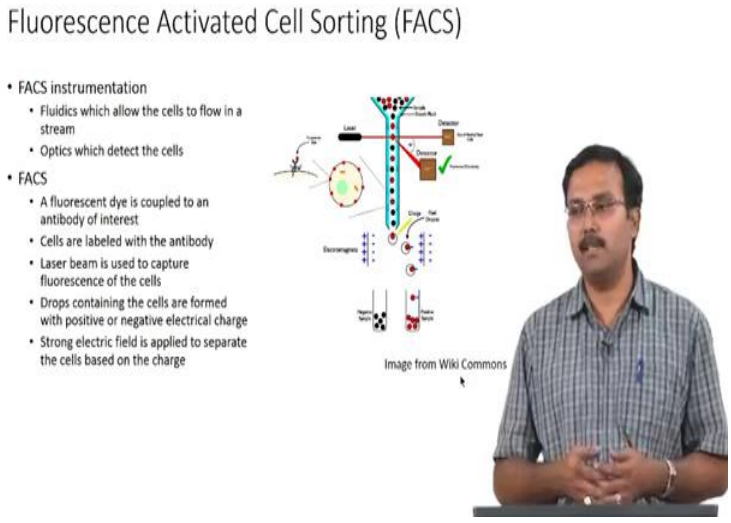

FACS has two major aspects when it comes to instrumentation. One is the fluidics aspect. So, the fluidics has to allow the cells to flow in a stream and ideally in a stream of one cell. It should be optimized in a way there is one cell will pass through these things. And the optics have to be there for it to identify and detect the cells. The FACS has a fluorescent dye, which is coupled to an antibody of interest, the cells are labeled with this antibody, and a laser beam is used to capture the fluorescence of these cells.

So, the drops which contain the cells will form positive or negative electrical charge, and a strong electrical field is finally applied for the cells to be separated based on the charge. So, this is the simple procedure that is followed for cell separation using FACS.

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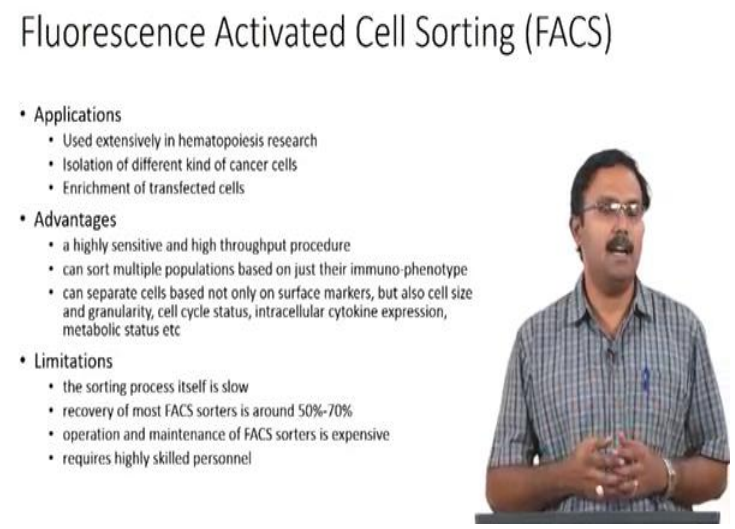

The application is, it is used extensively in hematopoiesis research; it is used for isolation of different types of cancer cells, enrichment of transfected cells, and so on. The advantages are it is highly sensitive, and it is a high throughput procedure; it can sort multiple cell populations based on just the immuno-phenotype. It can separate cells based not only on surface markers but also on things like cell size, granularity, cell cycle status, intracellular cytokine expression, metabolic status, and so on. It can be used for separating cells based on so many factors, which give it is a huge advantage.

The limitation is the sorting process itself is low, and the cell recovery is about 50 to 70%, and the operation and maintenance of these machines are quite expensive. This requires highly skilled and trained personnel who can operate this, so that makes it very

difficult to have for individual labs and so on. In our department there are a couple of these, two are there in the common equipment facility. And I think one or two are present with Professor Mahalingam. Maintaining it is quite painful, and operational costs are also quite high.

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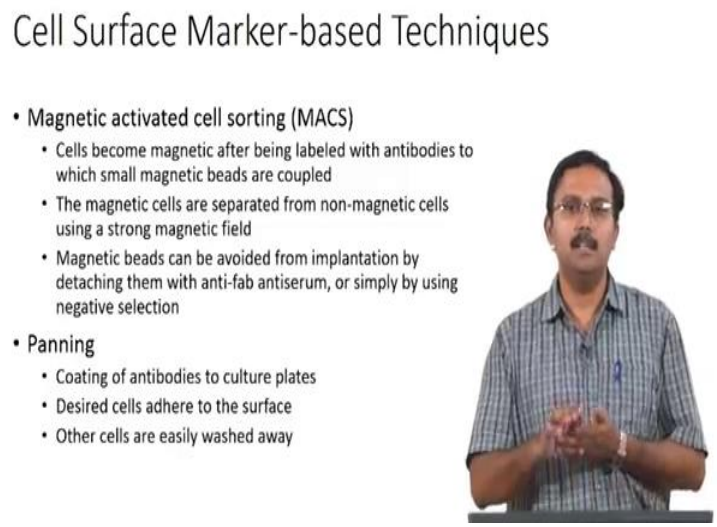

Other surfer marker-based techniques could be magnetic-activated cell sorting; instead of FACS, it's MACS and panning. In the case of MACS, cells are made magnetic by attaching some labeled antibodies, which will have some of these magnetic beads coupled to them.

And the magnetic cells are separated from the nonmagnetic cells using a strong magnetic field. Magnetic beads can be avoided for implantation; if you do not want these beads, detach them using some antiserums and so on, or you can also use negative selection. You just do not tag the cells you want and tag the other cell; then, you will get cells without any of these tags. Panning is a simpler technique; all you are do is, you coat antibodies to culture plates, and only the desired cells are adhere to, and other cells, which don’t adhere, are easily washed.

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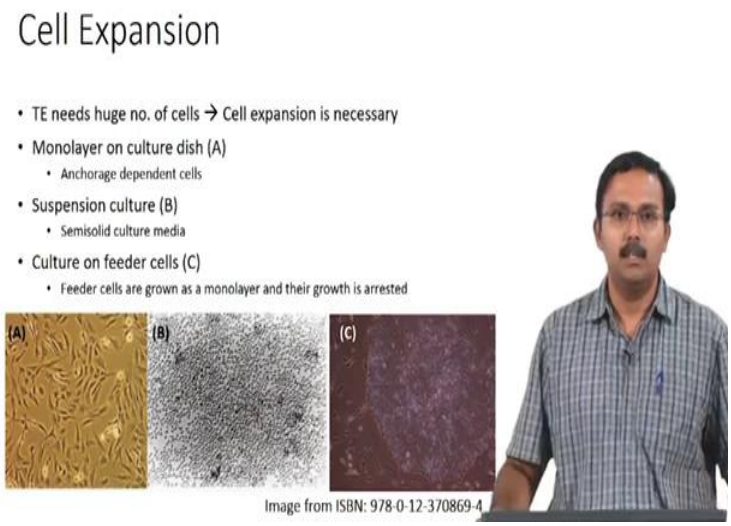

Whatever cells you are isolating would also have to be expanded. Tissue engineering usually requires large numbers of cells. And for cell expansion, there are different things that can be done. A monolayer on a culture dish is what is commonly done, which is 2D cell culture; this is done for anchorage-dependent cells. You can also have suspension cultures; suspension cultures are usually done using semisolid culture media. And you have culture on feeder cells; I think I mentioned this earlier as well. Feeder cells are grown as a monolayer on these culture plates, and then the growth is arrested. On top of the feeder cells, you culture the cells which you want.

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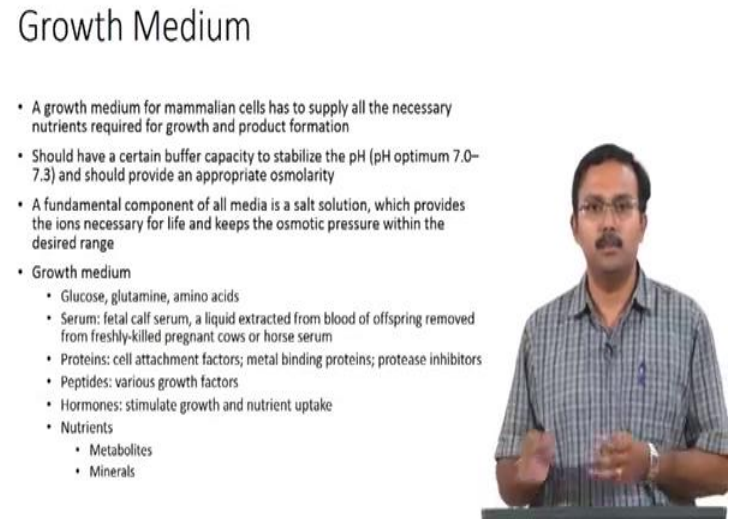

The growth medium you use has to be designed to supply all the nutrients. This would not be like what you have for bacterial culture, but it still has the same principles. You still need some carbon source, nitrogen source, some proteins, peptides, serum, which would basically contain different growth factors and other nutrients.

A serum, which is usually used, is fetal bovine serum, or you can also use it from horses. It is called fetal calf serum, which can be either from bovine source or from horses. You would be using peptides and hormones, and other nutrients such as minerals and metabolites might have to be added. This growth medium is usually complex; defined media is almost never used. So, you would have some kind of a complex medium. You would have things like DMEM, which have different things combined and with serum without serum and so on, depending on how you want the cells to grow.

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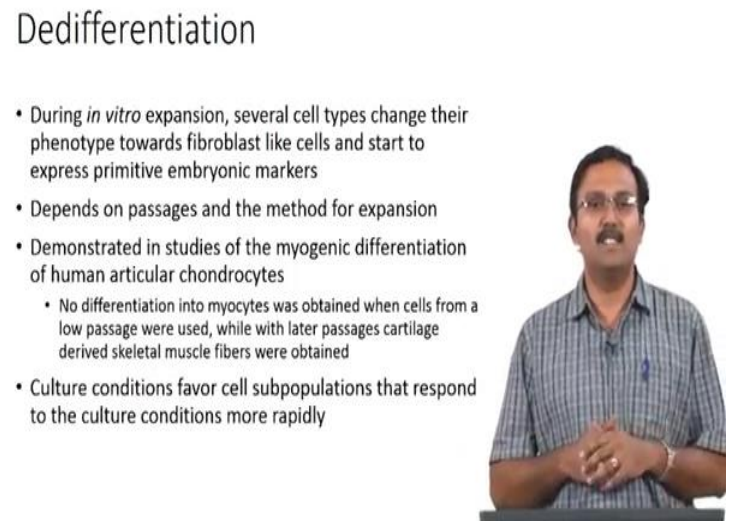

Dedifferentiation of cells is one of the factors which needs to be accounted for when you are talking about in vitro expansion. Several cell types can change their phenotype towards fibroblast-like cells while they are growing in these kinds of culture environments.

This depends on the passages, and the method used for expansion; the nutrients that you provide, the conditions you provide will direct them into different lineages. It can transdifferentiate into fibroblast-like cells, which is not a good thing; you would want the

specific cell types. There have been studies that have shown that chondrocytes can differentiate to form something like myocytes, myogenic cells.

When you have low passage cells, this kind of transdifferentiation or dedifferentiation is usually lesser, but as the passages increased, you would have some of these problems. So, the culture conditions can favor cell populations that respond more rapidly, and because of that, these particular cell types will probably move towards other cell types.