Introduction to Biomicrofluidics
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Lecture – 18
Microfluidic Technology for Monoclonal Antibody Production

So, far we have discussed that basics of cell biology, then we came across how that microfluidics platform could be used to adjust that mechano (Refer Time: 00:33) in cell biology. Then we have discussed how to perform that different formats of cell culture in 2 dimensional and 3 dimensional in microfluidic system. And we have also discussed about the various experimental procedures should be adopted in this same platform. After that we had gone through some important biological readouts in the cancer meta status area, mimicking that physiological micro confinement in this same platform which could not be assurgent by using macro cell culture.

Then we have discussed that organs on chip to mimic that physiology of human system and it could be used for drug skinning purposes, followed by lab on a chip model for facing DNA genes may be abnormal or normal genes out of millions of gene fragments DNA fragments. And same way to identify that proteins from that any biological sample using this microfluidic platform. And in my last lecture means this lecture I am going to discuss that how hybridoma technology could be adopted in microfluidic platform for the molecular antibody production. And before going to monochrome antibody we have to know about that what is antibody?

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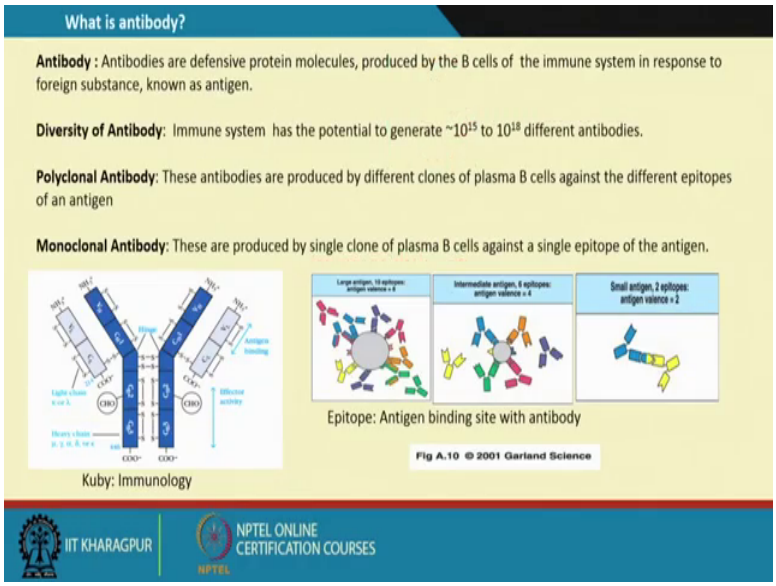

So, antibodies are defensive molecules produced by the B cells in a immune system in response to foreign substance known as antigen. So, in antigen say if you take an example this is an antigen it has a several epitopes and B cells in our body recognize most of the epitopes as an antigen fragments then B cell is differentiated to plasma cells then it produces antibody. So, like say this antigen has a 10 epitopes, this antigen has a 6 epitopes, this antigen as a 2 epitopes. So, according to clonal selection theory only 1 B cell clone will liberate only one type of antibody against one epitope.

So, if you inject antigen in our body or in animal system that our immune system will produced large number of antibodies which are polyclonal in nature. So, that is why polyclonal antibodies define in that way, these antibodies are produced by different clones of plasma B cells against that different epitopes of an antigen. Same way monoclonal antibody these are produced by single clones of plasma B cell against a single epitope antigens. And our immune system has a diversity to produce antibody in the range of 10 to the power 15 to 10 to the power 18 different antibodies.

And here is that picture of that main class of antibody immunology in G I G G basically. So, this molecule has a heavy chain this is the heavy chain and this is the light chain and this part we known as fav part which actually recognize that antigen epitopes specifically and with high binding constant and this part is known as FC part means constant part.

And if you are interested in immunology you can go through that Kuby's book on immunology I am not going to in details.

Now, how to produce monoclonal antibody in vitro? Because, monoclonal antibody has a diverse use starting from diagnostics to modern biology lab for identification of a protein from that vast large amount of proteins using histo chemistry or western blots antibody based microarray system and after all lot of therapeutics are coming based on that antibody.

So, now how to generate that antibody? These B cells which are generated in BO; these are not very viable they will eventually it will die after that secreting antibody. So, if you want to produce antibody from the B cells, we have to immortalize the B cells in vitro. So, how this could be done? So, this was addressed first by Kohler and Milstein in the year of 1960s.

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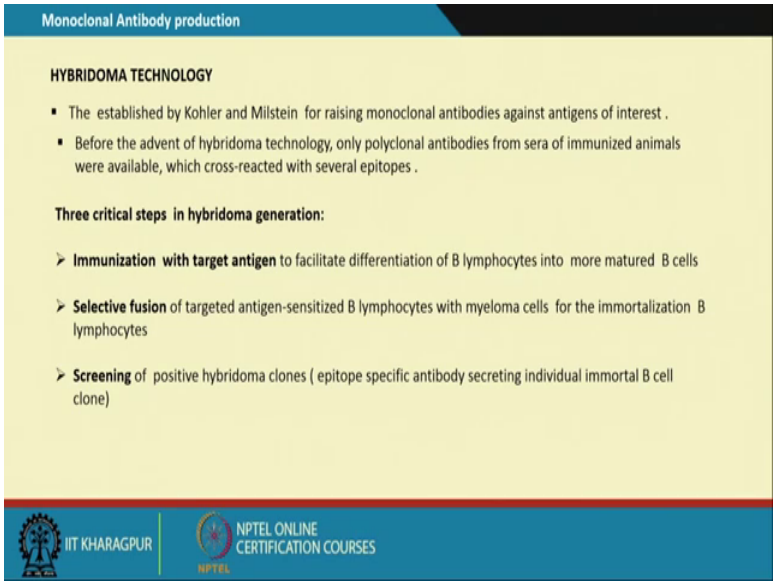

And they developed that hybridoma technology; basics of hybridoma technology is that fusion of B cells, antibodies secreting B cells with a cancerous B cells in myeloma cells, then select that is selection of the fuse itself that is called hybridoma means oma means it is a cancerous cell.

Then you have to screen that positive hybridoma clones epitope specific antibody secreting individual immortal B cell clone. So, basically for the development of

hybridoma 3 steps are there immunization with target antigens, selective fusion and then screening. Then out of 10 to the power 7 cells from a my spline how will you select single B cell clone for that monoclonal antibodies?

A tremendous task so you have to adopt some selection procedure by which that only that hybrid cell means one B cell one myeloma cell that fused hybridoma cells will be selected; so, how to select this? So, this is the problem and it was discover by that Kohler and Milstein in 1960s.

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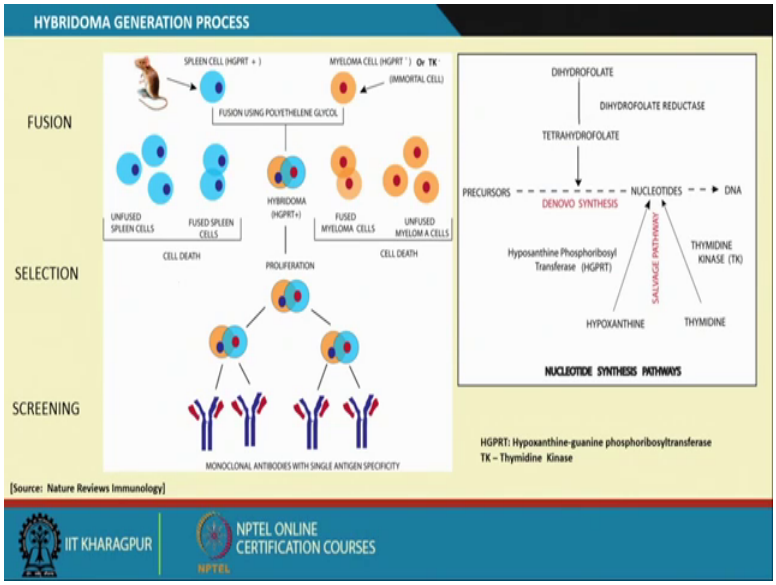

So, that principle is that if we can block that one of the major important molecules by synthetic path way like say nucleotides then cell will not divide cell will die. So, that nucleotides are synthesized by 2 pathways one is denovo pathways from the precursor and one of the important enzyme in that pathway is dihydrofolate reductase.

And another pathways salvage pathway which utilize that hypoxanthine and thymidine your nucleosides for that DNA synthesis. If you can block this two pathways then one cell will die, but here our target is you want to die that myeloma cells, but hybridoma cells will survive. So, when you are fusing after evenization we take the spleenocites around say 6 into 10 to the power 7 cells and myeloma cells around 2 into 10 to the power 6 cells; mix up you are giving fusion mixer with the polyethylene glycol for certain period of time 15 minutes.

Then you will be seeing that there are 5 groups of cells, this is the our required cell hybridoma cells along with fused spleen cells, unfused spleen cells, fused myeloma cells and unfused myeloma cells. Our requirement is one fusion; one B cells (Refer Time: 08:37) B cells activated B cells and another myeloma cells and frequency of that fusion around one percent of that total cells we have used. Now, how to select these? As I have already mentioned that DNA synthesis blocking of the DNA synthesis could be one of the best strategy for the selection.

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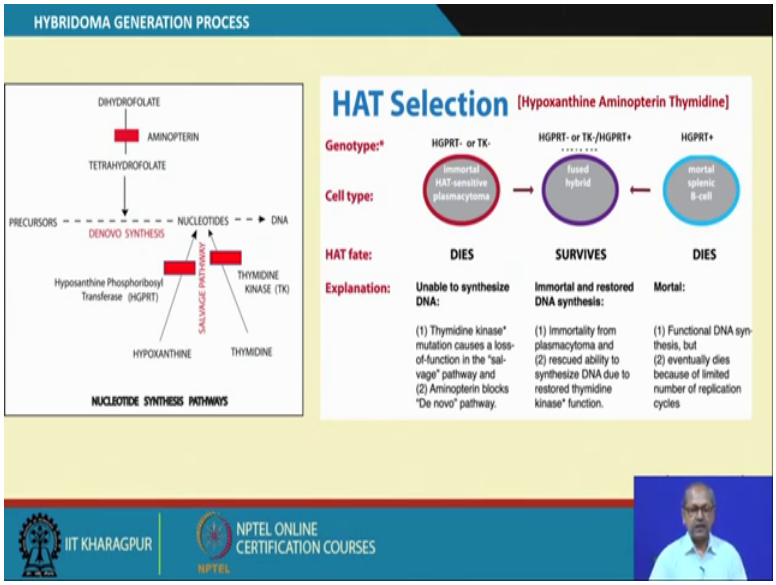

For this hat selection media is used what is the composition of the hat selection media? Hypoxanthine, aminopterin and thymidine. What is the function of aminopterin? It will block that dihydrofolate deduct as enzyme by is denovo synthesis blocked and as we are selecting that myeloma cells, which are HGPRT minus or thymidine kind of minus they have a already that blocked that salvage pathway; means in that cocktail of that fusion pot in that mixer of hat selection media that your mortal splenic cell means die your splenic B cells will die due to that 2 or 3 generation of culture whereas, HGPRT minus or TK minus immortal hats sensitive plasmacytoma will die.

So, only it will survive on 2 or 3 passages or 3 generation of cell growth that fused hybrid cell or hybridoma. This is that basic means technology was discovered around 1960s and that is still follows for the hybridoma production.

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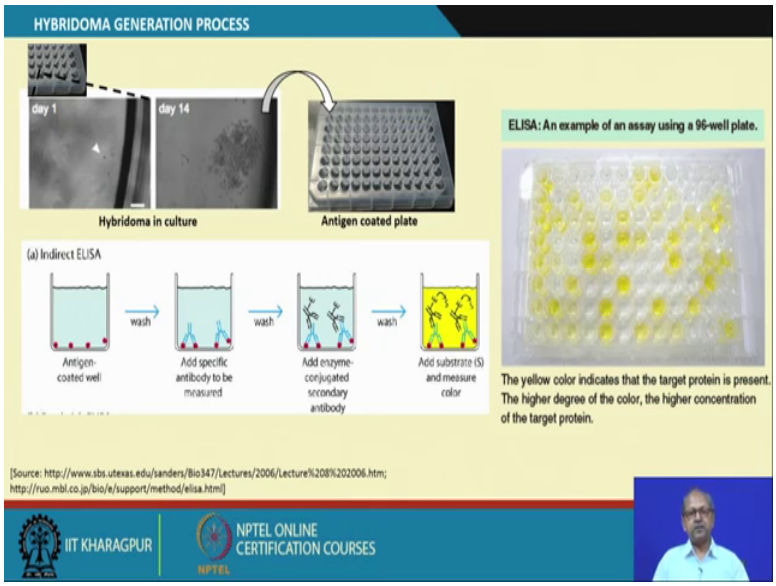

So, next step is how to screen that effective hybridoma cells for the require antibody which will be monoclonal in nature. So, we have to by dilution plating that hybridoma cells will be plated in 96 well plate by we which one one hybridoma cell will be in one one well. Then let it culture for 1 to 14; there is according to the need then cell will be growing you can see under the microscope. Then you have to test that culture fluid for the antibody what we need which will be in monoclonal in nature because these are derived for 1 B cell.

So, for that we have to use indirect antigen system say take for an example this an 96 well plate is coated with that antigen what we have being injected in the mice, then we are giving some blocking reagent then we are giving 100 microliter of that your culture fluid from that each well to that antigen coated plate. Again you was throw it away then you give that anti body means FC portion against the antibody against that FC of that antibody secreted tagged with reporter enzyme let it bound. Again you wash it then you give some subtract for that tagged enzyme by which colour will be produced substrate to product converse.

Then will be seen like that if this is the replica of 96 well plate of these plate then only you will be seeing that only some plates will be seeing the color means these are the positive clone hybridoma clone which are producing antibody. From that you have to again screen it because hybridoma cells are heterotopic fusion these are not very stable to

make it a stable hybridoma cell line, we have to another selection of 2 or 3 times you have to go through by which will be getting stable cell line and you have a stable hybridoma cell line which will be secreting antibody, but keep in mind these are not very stable for long term.

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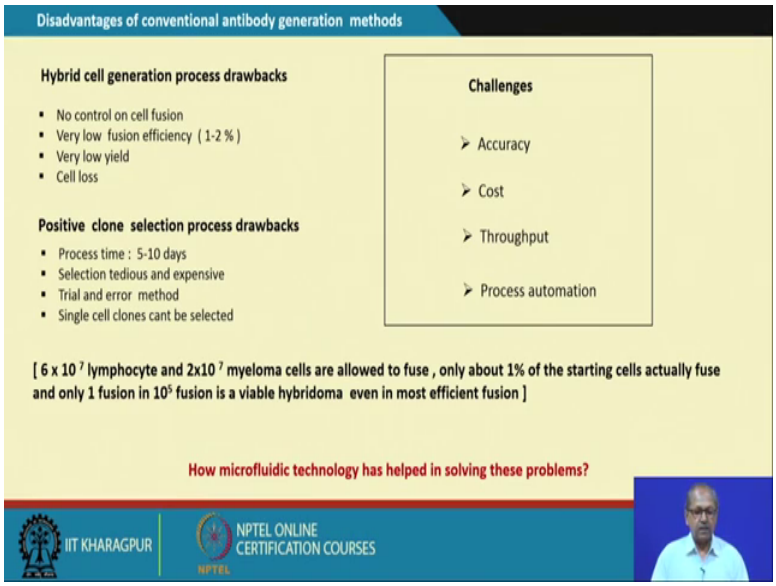

So, what are the drawbacks and challenges of this macro cell hybridoma cell culture or hybridoma generation? There is no control of fusion, very low fusion efficiency around 1 to 2 percent very low yield cell loss. How much time is required process time around 5; 5 to 10 days selection is tedious and expensive and single cell clones cannot be selected in the initial stage.

So, challenges are accuracy, cost, throughput and process automation. So, this is one of the data means if we give 6 into 10 to the power 7 lymphocytes and 2 into 10 to the power 7 myeloma cells are allowed to fuse only about 1 percent of the starting cells actually fuse and 1 fusion in 10 to the power 5 fusion is a viable hybridoma even in most efficient fusion.

So, how this these problems could be addressed? We think the microfluidic technology is the best technology to address all these problems that is means fusion and screening process in high throughput way within a very short period of time by which we can select a positive hybridoma clones for secreting one class of antibody what we can tell is a monoclonal antibody.

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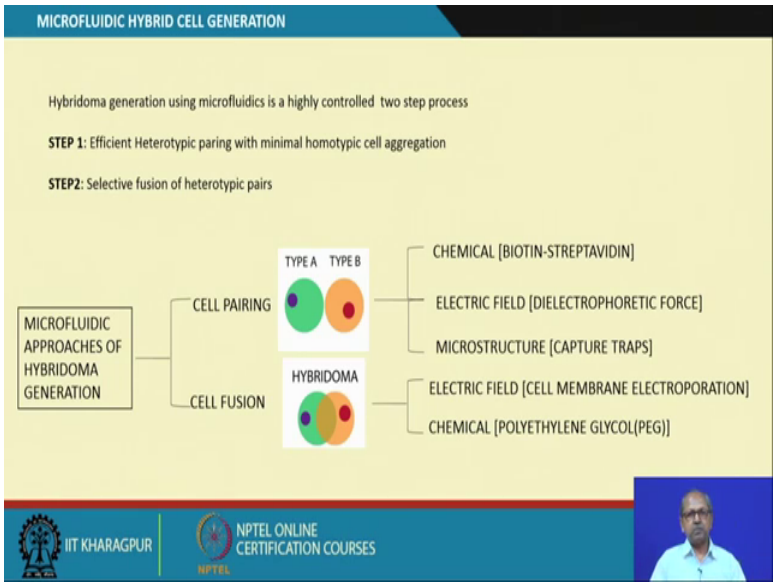

See if we use microfluidic pattern what are the types or what are the problems we can address? So, first problem is that or first step you can tell that efficient heterotypic paring with minimal homotypic cell aggregations means myeloma myeloma or B cell B cell only we want that heterotopic pair pairing that is cell pairing type and type B this is myeloma this is your B cell and then selective fusion of heterotypic pairs then next is screening.

So, first we are coming cell pairing and fusion method in fluidic platform; this could be done by chemical ways electrical field, dielectrophoretic force and microstructures. And fusion could be done by this is chemical way that is a what we followed in macro scale, then electrical field cell membrane electroporation method that could be done.

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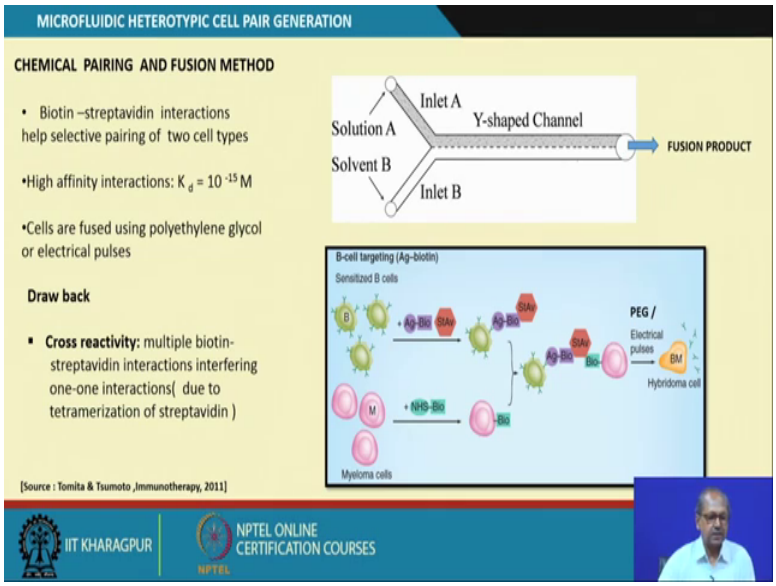

First we are coming that chemical pairing and fusion method; here principle is adopted that streptavidin selection process these are a very strong ligand and receptor there are binding constant around 10 to the power minus 15 molar. So, myeloma cells are lathered with biotin normal chemistry is there and B cells or you can splenocytes are labeled with streptavidin.

Now, with the help of Y channels in fluidic platform in one channel say B channel we are giving myeloma cells and A channel we are giving labeled B cells; then allowed to flow in Y channel and at certain distance let it come together and with a micro mixer, they will be mixing and fusion using either electrical method or PEG using polyethylene glycol.

But problem is that cross reactivity multiple biotin streptavidin interaction interfering the one one interactions what you want one one paring it will be there, but not very high percentage.

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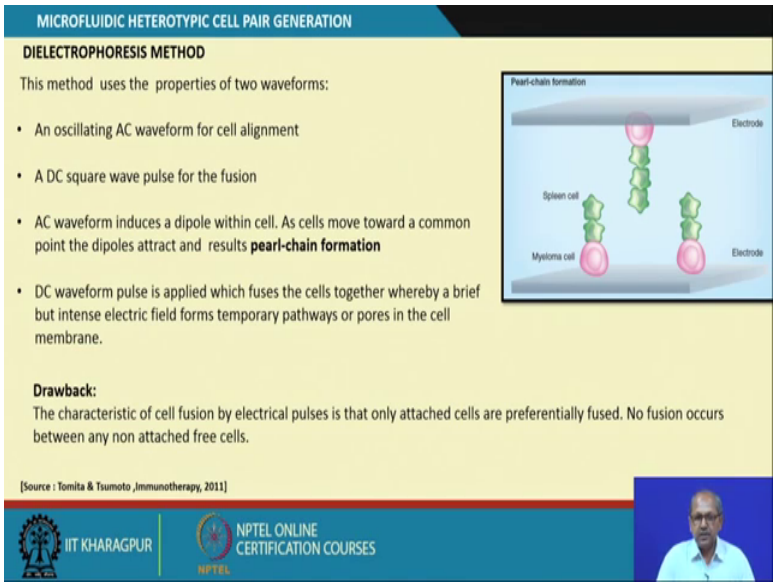

If we use dielectrophoresis method; these are the electrodes and first we are passing one type of cell say myeloma cells, they will be clustered around the electrodes then we are passing that spleen cells activated spleen cells and they will be clustered around that myeloma cells and there will be pearl chain type of formation will be the cells.

So, here is also that only one one pair occurs not very large extent and then you can use pearls DC field for a few microseconds or 1 second to fuse the cells and o off that dielctrophoresis; then you can get that fused cells, but their percentage is not very high due to that streptavidin hybrid in because streptavidin is 4 titrimetric complex they also make a cluster.

So, next is that how that microstructure could be utilized in microfluidic platform to capture single cells, then pairing with 2 cells one one pair then you fuse.

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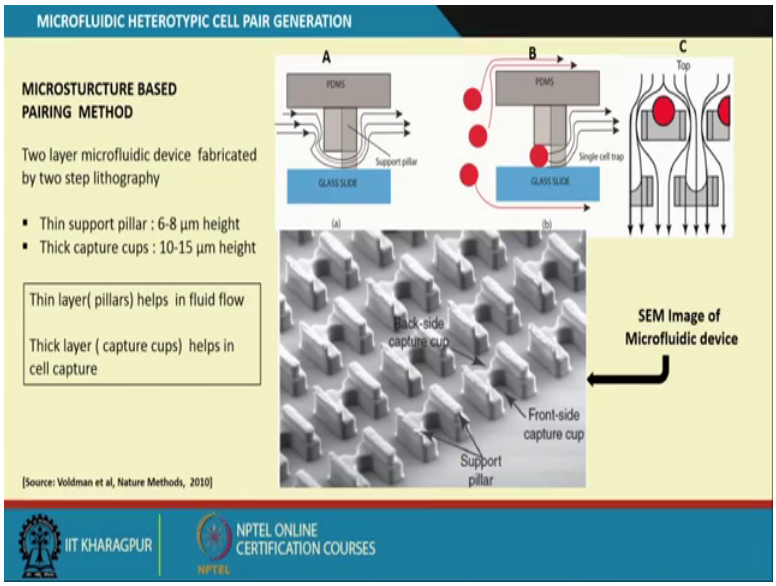

This is very beautiful structures means designing with respect of design and that flow we are of that cells in that microfluidic chip. So, here principle is that you have 2 layer microfluidic device fabricated by 2 step lithography; this is your support pillar and this is a capture cup.

If you look for the capture cup these are 2 sided one is front side one is back side; in the front side there is notch of diameter around 10 micrometer, in the back side cup diameter is around 20 micrometer. And that support pillar will help to fluid flow and that cell will be captured first in the front cup, then it will be flowed in such a way that will be going back to that means, from this place to that front that larger cup then again you give it; so, this is demonstrated in that ok.

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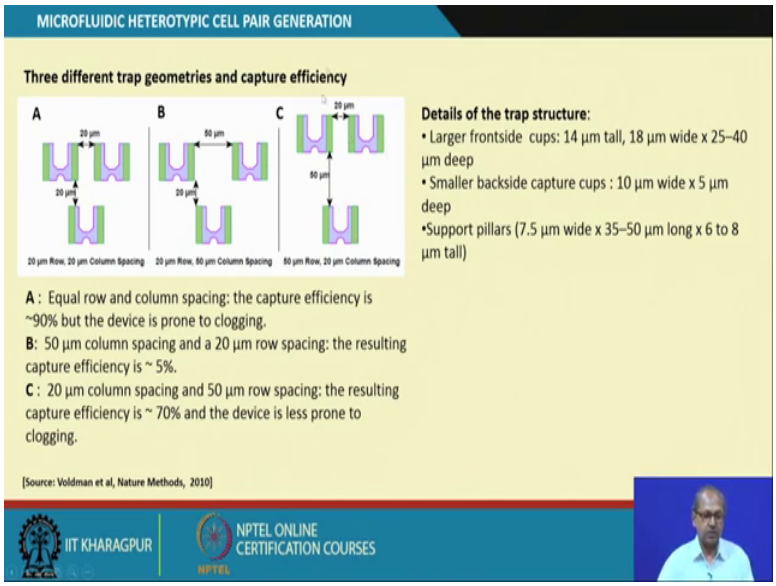

So, this is that configuration of that cups and this is the distance means; if this 2 cups at that aligned around 2 micrometer spacing and 50 micrometer low spacing that is the most desired cup alignment in the microfluidic platform, which allows around 70 percent of the capture that cells.

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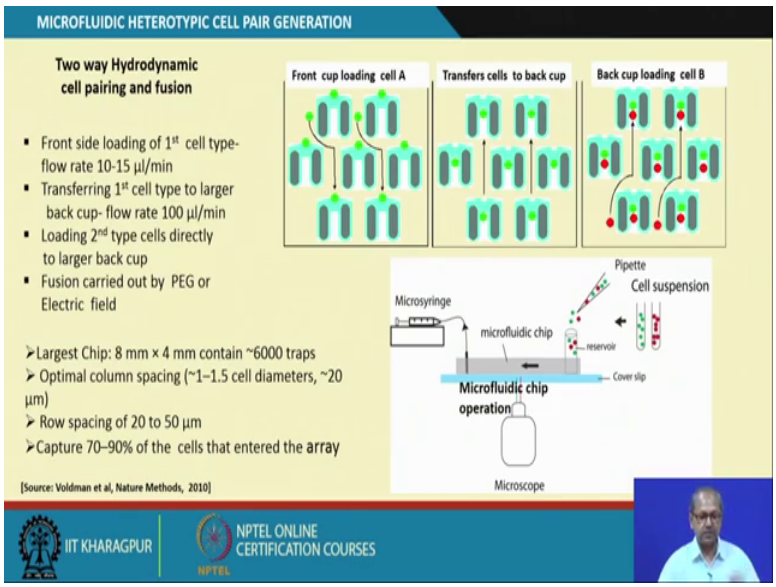

Now, how cells are capturing? First that one type of cell like say myeloma cells we are flowing in the direction at the rate of 10 to 15 micro liter per minute, then cells will be

captured in that small cup of diameter is around 10 micrometer so, one one cells is captured.

Then we are giving opposite direction flow around 100 microliter per minute by which this captured one cells say myeloma cells will be transported to the B go well of that just opposite microstructure. So, that myeloma is captured; then you give the B cells in the same directions then B cell will be entering in that well like say B cells are entering then one one capture will be there.

This very idealistic picture, but in a real situation what you observed that there are some clusters are there mean 3 cells are coming together, but their percentage is less. So, in that way around 70 to 90 percents cells will be captured just one one pair.

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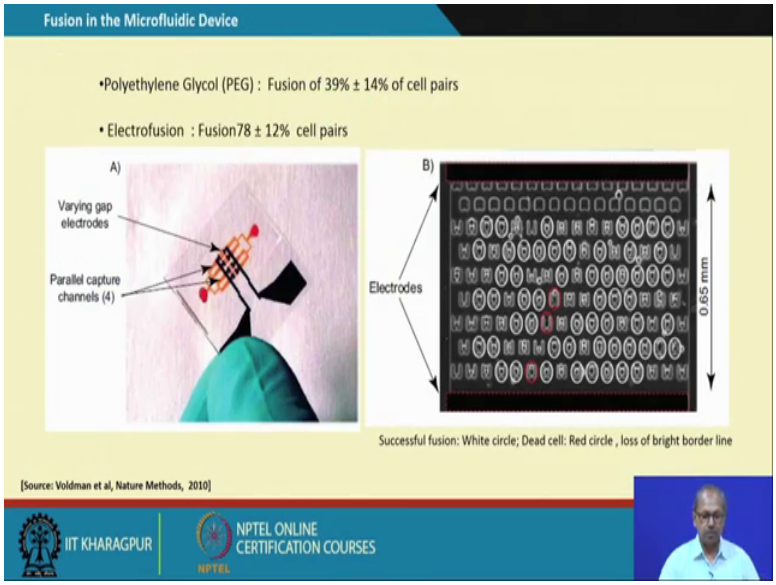

So, next is how to fuse that cell using that same platform that could be done using polyethylene glycol; fusion efficiency around 40 percent and if we give brief DC electric field for a 1 second or 2 second, then fusion efficiencies 78 percent. And both the things could be done in same platform because PEG here PEG is very easy to get because, we can quickly pass out that used PEG using that flow behavior in that microfluidic platform.

And this electric field in the beneath the electrodes are there you can give them directly active field. And this is a picture to showing that which ate the hybridoma cells are

survived and which are not survived; which are survived there is a clear cut demarcation of the periphery or is that dead hybridoma cells that is no clear cut demarcation they have they are dead hybridoma cells basically.

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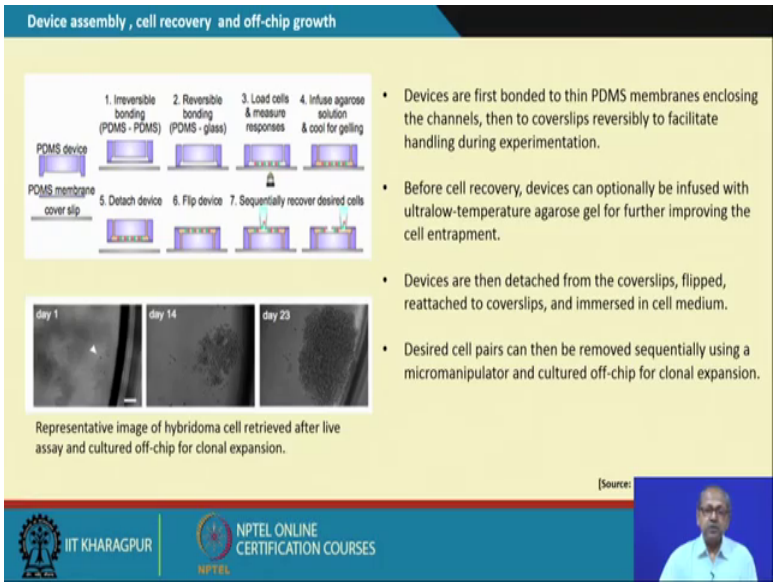

Now, next step is we have to capture that hybridoma cells which are fused inside that microfluidic chamber. For that we have to use that micromanipulator that operation is like that when you are constructing that PDMS device that this is the cover slip, this PDMS membrane this the PDMS device or the microstructures is there.

Then this PDMS membrane irreversibly bonding with upper PDMS chamber is where that inlet outlets are there, then the whole the structures is reversibly bonding with the glass plate then you introduced all the cells and everything what you have discussed right now.

Then if you want after that fusion you can introduce very low melting agarose for the gel setting to by which that fused cell will not be that much disturbed. Then you invert it like this position then with the using micromanipulator sequentially you can recover each and every cell and you collect in the microfluidic that micro well platform and let it grow.

So, like say this is an one example in the micro well this is a blow up picture this is the one cell and you are brewing for according to that there we are seeing that 14 days your growth, then again it will grow 23 days which are viable hybridoma cell. It does not

indicate that viable hybridoma cells will produce that antibody. So, you have to again go for that selection of the antibody selection means how to screen that so, many hybridoma cells for that antibody screening. So, this also could be adopted in microfluidic platform.

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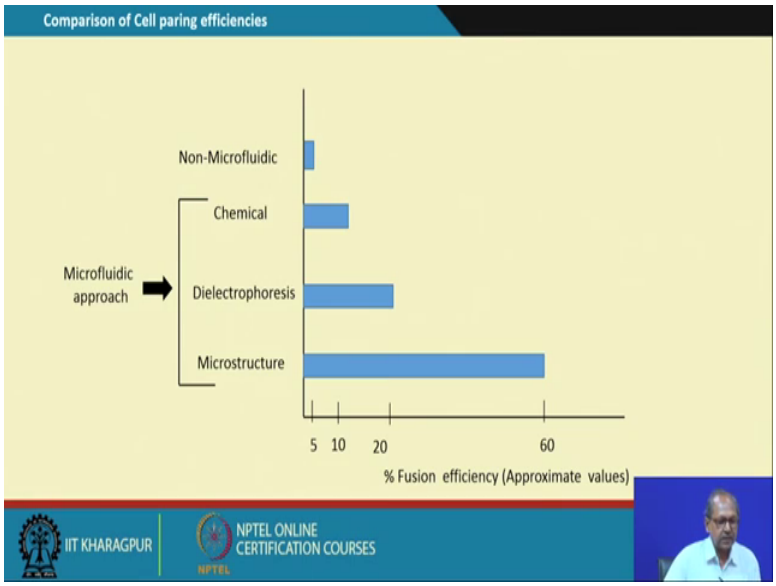

So, if we look for that cell pairing efficiency of this 3 approaches or 4 approaches; non microfluidic means macro cell culture this is the 5 percent chemical way around say 15 percent, dielctrophoresis around 20 percent microstructure around 60 to 70 percent.

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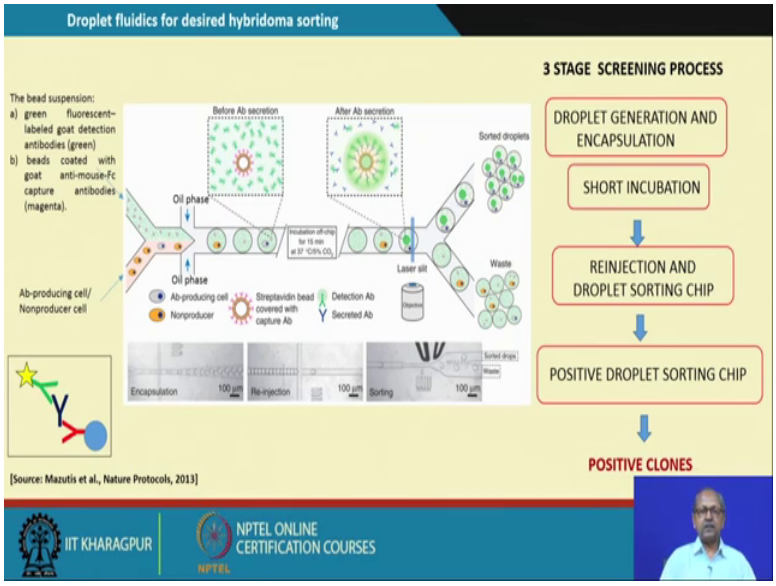

Say as I mentioned that next step will be screening of that hybridoma cells for that antibody secretion means positive clone. So, before going to that experiment you have to select how to screen that antibody; how to means experimentally observe that anitbodies secreted and as say that antibody.

Here that technique is like directlize that what you have done in earlier that is macro cell process; here principle is that say this is the secreted antibody this is blue in color and we have that capture antibody; this is red color which is against that FC portion of that antibody ligated with that micro bead. Then detection antibody which is against that FAV part of that secreted antibody in got labeled with fluorescence.

So, principle is that when that secreted antibody from that hybridoma cells they will react with that first that your detection antibody, then that will be captured by that bead containing high density capturing antibody, then there will be a glow of fluorescence around that micro bead. So, that is the basic principle of detection now how will you screen it?

Here screening is done by droplet based fluidics approach in the this is the Y channel this is the one channel that bead suspension what we discussed right now is given. And another channel you are giving that hybridoma mixture of is positive clone and negative clones. Then you are flowing that 2 aqua solution and using oil phase chlorinated silicon oil phase that using flow focusing method pinching that aqua spot in a droplets.

So, these droplets are in such a size that you can capture the only one cell along with beads. So, there is a probability of 3 types of beads 3 types of means capture tubes sorry not capture tubes is a 3 types of oil phase encapsulated. This one is only beads and some your detecting antibody and one is which captures that antibody secreting hybridoma cells, another class will be a non secreting that 3 class of droplets will be there. So, after that passing that droplet you capture the droplets in a pen drop tubes and let it stay for another 1 or 2 hours may be 3 to 4 hours to secret that antibody from that hybridoma cells.

Then the next step will be screening that antibody secreting hybridoma cells means which are the positive clone and negative clone. As we are incubating in due to incubator for certain time, antibody will be produced and that antibody will react with that your detection antibody; eventually it will be captured by that beads and fluorescence will be

concentrated in the beads. So, those will be screened by using laser slit and dielectrophoretic method by which that positive clones means which are secreting antibody which are fluorescence positive bright fluorescence positive they will be captured and sorted in the droplets and which are not producing antibody, they will be wasted.

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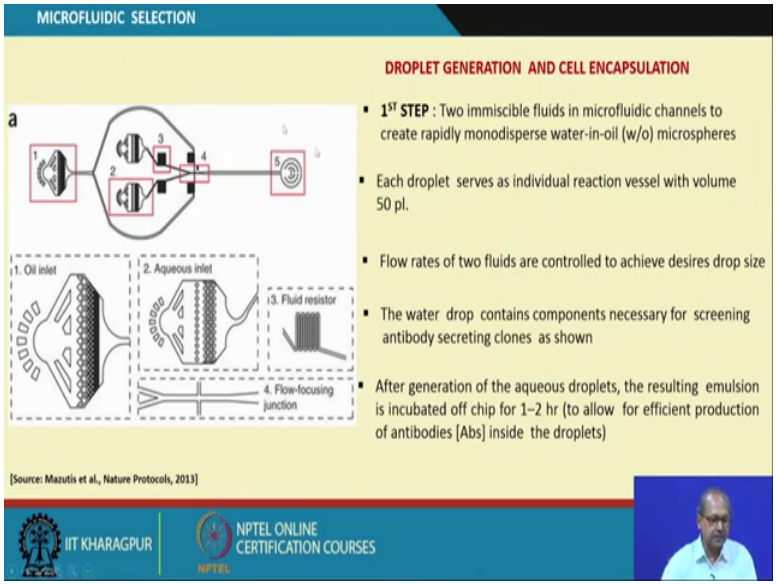

So, this is the in details of that your droplet generation and cell encapsulation process. This is 2 and 3 these are the aqueous compartment which one compartment containing that your mixture of beads and that fluorescence antibody, then another compartment containings that all the hybridoma cells.

And this compartment 1 containing the oil phase fluorinated silicon oil phase and when this 2 are coming 2 phases are coming at that focus at this point; then oil phase pinch that aqueous phase make the droplets of the required size. And size of the droplet depending upon that what speed of that all the 3 solutions and it should be standardized by which the droplet size will be such a that it can encapsulate at least one one cell. Then this is the capture where the cells are captured and this is the flow focusing happening here by which droplets are generated.

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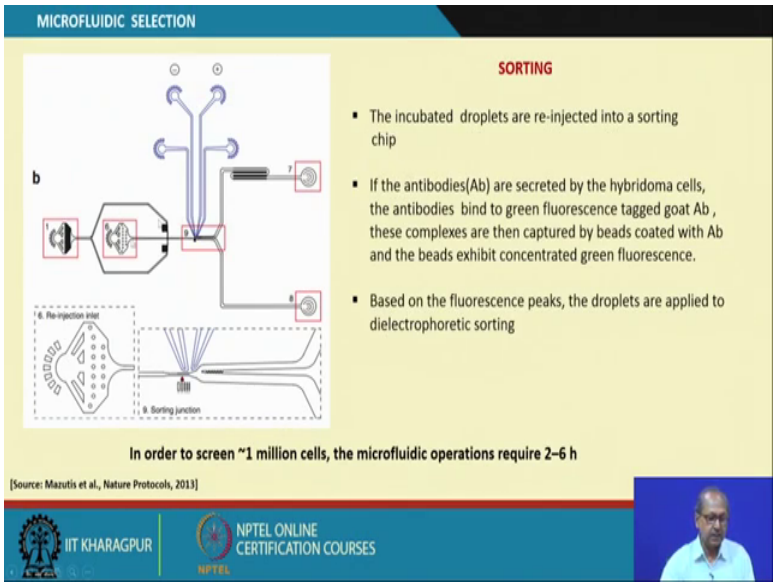

So, these are details of that screening. So, next step means when that cells are stored here after 2 or 3 3 to 4 hours according to the need let it allowed that antibody secretion and some droplets will be glowing some droplets; majority droplets will not be glowing. Then same way again flow focusing is done it in that place; let it pass through that your screening chamber laser and dielectrophoresis setup and this is the cells are sorted and which are the not fluorescent, they are the going out.

So, in that way we have we can screen around 1 million of cells in a microfluidic operation 2 to 6 hours. So, now if you can compare that 2 system means macro scale and your fluidic system we have a lot of leverage in micro scale system.

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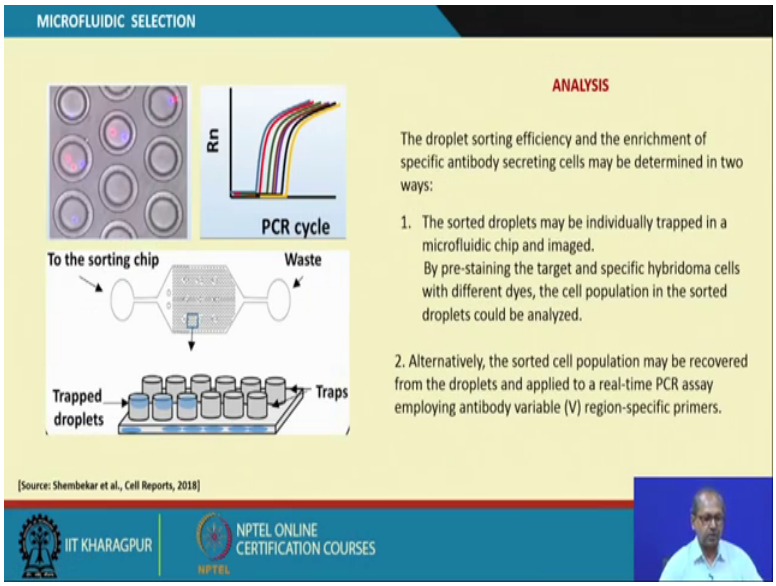

After selecting this positive clones, to assure that these are the positive clones the sorted droplets may be individually trapped in a microfluidic chip here that microfluidic chip and it will stand to target specific hybridoma cells with different dyes. Otherwise, you can so PCR cycle for the FAV part of that antibody, they can look for that whether it is a positive is selected. So we can save the time as well as monotonous process for the screening by it takes might be 2 to 3 months if you are lucky and may be end up with 2 or 3 clones of that monoclonal antibody secret in hybridoma.

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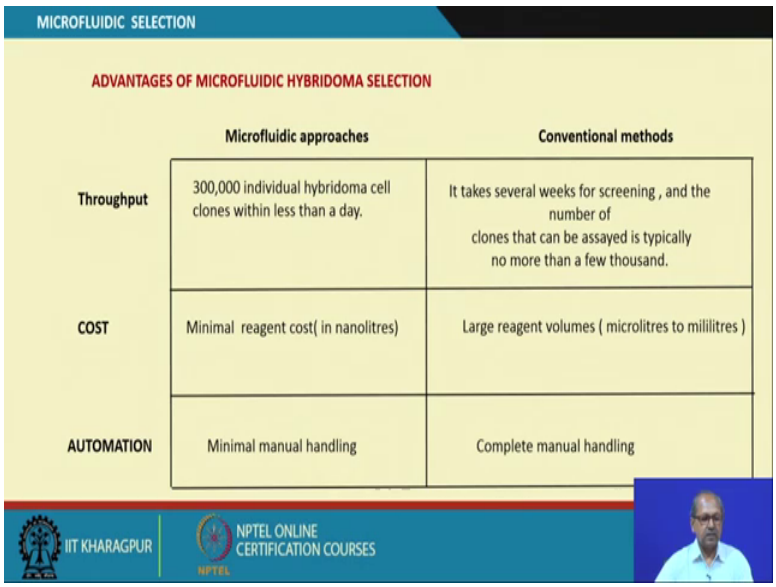

So, if we can compare that throughput cost and automation in each of the compartment microfluidic has a higher leverage than conventional method. So, nowadays people are using that molecular biology technique by which we can screen that monoclonal antibody secreting cells fetching that your sequence of that fav part, then clone it then using path (Refer Time: 31:10) technology. And using that microfluidic technology large scale screening could be done with a very short period of time that I am not discussing here.

But, anyway microfluidic technology has a better impact or the screening process and selection process is preferred rather than normal macro scale process. So, I think with these 10 lectures I shall be able to some idea about that microfluidics cell culture system and some readouts which are not possible in macro scale system; by which we could be inspired to adopt microfluidic cell culture in our day to day cell culture practices for two reasons; one for discovery of new readouts, which are not possible in micro scale macro scale cell culture system, another thing application aspects which has a huge impact in our modern biology.

Thank you.