Cell and Molecular Biology
Prof. Vishal Trivedi
Department of Biosciences and Bioengineering
Indian Institute of Technology, Guwahati
Week 03
Cellular Homeostasis
Lecture - 12
Cell Cycle

Hello, everyone. This is Dr. Vishal Trivedi from the Department of Biosciences and Bioengineering, IIT Guwahati. And in this particular module, we are discussing cell growth and cell division. So for what we have discussed, we have talked about the importance of growth and how growth is regulated within the prokaryotic system or the eukaryotic system. In this context, we have discussed the cell checkpoints and how cell growth is regulated by following the different stages.

Each stage of cell growth is going to be regulated by cellular machinery or a set of proteins. Subsequent to that, in the previous lecture, we also discussed the different types of events, what is happening within cell growth, and how you can provide nutrition to the cellular system. So we have discussed the preparation of the media for the prokaryotic system, and subsequently, we have also discussed the different types of methods for monitoring the growth of both prokaryotic and eukaryotic cells. Within the eukaryotic cell, we have discussed the monitoring of growth for the animal cell as well as for the plant cells.

So now it is clear that growth is very important for the longevity and survival of living organisms. And the growth is being divided into different stages. Right. And all these stages are part of the cell cycle. Right? So when a cell is growing through the different stages of the cell cycle, it actually follows a very regulated manner.

And that's how it is actually going to be accomplished. The purpose of cell division is to ensure that it does not give rise to abnormal cells or any kind of abnormality. So when you talk about the cell cycle in the eukaryotic system, the cells undergo different stages. And these different stages are being regulated by the cyclins, CDKs, and all those different molecules that we have discussed in this particular course. So in today's lecture, we are going to discuss the cell cycle and how it can be studied within the biological system, as well as how you can study the different stages of the cell cycle.

So, the cell cycle is, as we say, right; the cell cycle is being done in the eukaryotic system. The eukaryotic cell undergoes a precise cell cycle and division to produce two daughter cells. The cell cycle is a series of tightly regulated events leading to division and duplication. It is a vital process used for a single fertilized egg to develop into a whole organism. The cell cycle is the crucial event underlying regeneration and repair in tissues, such as the liver and heart.

This is very, very important. And this part I think we have discussed when we were

discussing the first lecture, right? That's why cell growth is required; it is necessary so that you can replenish the damaged cells, and it will also help bring about the repairing process, right? In the prokaryotic system, the parent cell divides simply by binary fission, and that's why the cell cycle is not that relevant in terms of the prokaryotic system. But even then, we have discussed that in the prokaryotic system, you also have different stages like cell growth; then you are going to have the synthesis of the DNA, and then you are going to have the division, right? Whereas in the eukaryotic cell, there are three different stages in which the synthesis of genomic DNA occurs in the cytoplasm; then, you have the mitotic phase, where the division of DNA into two halves takes place, followed by cytokinesis. So these three stages are universal, whether it is a prokaryotic system or the eukaryotic system. In the prokaryotic system, because the cells are very small and the system is very simple, we don't divide that into different phases.

But in the prokaryotic system, you also have the three different stages where you are actually going to have the synthesis of cytosol and the synthesis of genomic DNA. Then you are going to have the division and distribution of the DNA between the two halves of the cells, and then there will be cell division by cytokinesis. So these are the three stages: you are actually going to have the G1 phase, where the cellular machinery is going to synthesize the different types of biomolecules that are required for DNA synthesis. So you are going to synthesize the DNA polymerase, you are going to synthesize the nucleotides, and all that. Once the cell is ready, it is actually going to cross from G1 to S phase, and then it will enter the S phase.

Within the S phase, it is actually going to do the DNA synthesis. Once the DNA synthesis is over, it is actually going to enter the G2 phase. The G2 phase is actually going to synthesize all the machinery required for cell division, and then it will enter into mitosis. After mitosis, once the cell divides the DNA between the two daughter cells, it will perform cytokinesis so that the cell divides into two parts: one is the mother cell and the other is the daughter cells. During the cell interface, you have two different phases; one is called the interface.

Within the interface, you have the G1 and G2 phases, and then you have the M phase, which is the mitotic phase. In the interface, the preparatory phase is required to perform the requisite steps; these are the series of events in the nucleus as well as the cytosol of the daughter cell to enable it to enter the division phase. These phases have several other phases, such as the G1 phase, so it is also known as the growth phase. So it starts from the end of mitosis and continues until the beginning of the S phase. This means during this phase; it is actually going to start by the end of the M phase and then end at the beginning of the S phase.

During this phase, the cellular protein enzymes are synthesized. What enzymes are going to be synthesized? The enzymes that are going to be synthesized, which will participate in the S phase, such as DNA polymerases, helicases, topoisomerases, single-stranded DNA binding proteins, gyrase, and all those kinds of cellular machinery. All this machinery and their detailed discussion will actually be addressed when we discuss the central dogma of molecular biology, where we will go into detail about DNA

applications. So most of these enzymes or the protein components are required for DNA synthesis in the S phase. The duration of the G1 phase depends on the cell type within the organism.

So the G1 phase is under the control of the P53 gene product. And remember that when we were discussing the lecture number one, we discussed in detail how P53 regulates the cell cycle and how P53 activity suppresses tumor activity. So it is actually because p53 is controlling the cell cycle in such a way that it is not allowing, you know, the rapid duplication of the cells, and it always wants the cells to go through the division phases under tight control. Once the G1 phase is over, it is actually going to enter the S phase. And once the cell grows and all the factors, like what are these factors? The synthesis of DNA occurs very quickly to avoid exposing newly synthesized DNA to mutations.

And this is very, very important that when you are synthesizing the DNA, it has to be done in discrete steps, right? It has to be done in a phase-wise manner, and on the other hand, you are not going to open the complete DNA. For example, if you have genomic DNA like this, right? Then, this is just an example: suppose you have this, so you are actually going to unbind this DNA, and then you are going to perform the replications. Then you are going to unbind this DNA and then you will do the replication. Then you are going to unwind this DNA and replicate it while you are doing the replication of this part; this part is already going to be closed, this part is going to be closed, this part is going to be closed, and then you will enter into the next phase so that a very small amount of DNA is going to be open and exposed to the environment, and that's how you are actually going to ensure that the synthesized DNA is free of exposure to mutagens. Then once the S phase is over, it will enter the G2 phase, the growth phase between DNA synthesis and mitosis.

During this phase, the cell grows and synthesizes the proteins and the cellular factors required for the M phase and cytokinesis. So once the G2 phase is over, it will enter the next phase, which is called the M phase. M phase is actually distributing the DNA between the sister chromatids. So after the G2 phase, the cell enters the M phase to divide the DNA equally between the two daughter cells; each mitosis has four distinct phases to precisely divide the cell. So during the M phase, you have two options: one, you are going to do mitosis, or two, you are actually going to do meiosis.

Mitosis is going to divide and distribute the DNA between the daughter cells, whereas meiosis is also called reduction division. So here, the DNA is actually going to be further reduced. So, you are actually going to reduce the ploidy of the cells. So if you started with 2N, it will enter meiosis, and after meiosis, it is going to be N, so that all the cells are actually going to have the N-ploidy. So we will discuss mitosis and meiosis, and you will understand the significance of mitosis and the significance of meiosis.

Apart from this, you also have another phase which is called the G0 phase. So after the G1 phase, some of the quiescent, senescent, and non-proliferative multinuclear eclotide cells enter the G0 phase. So the G0 phase is a non-dividing phase, right? So many of the cells that are in the terminal stage, which are actually not being allowed to divide and are

under the quiescent or senescent stage, will enter the G0 phase. Cells remain in this phase for a long period or an indefinite period; as in the case of neurons, it is also common in fully differentiated cells. The fast-growing cells enter the G0 phase, which is not a regular cycle, and under specific conditions, cells enter the G0 phase.

Therefore, whatever the cells are that enter the G0 phase, they will not divide right now. How are we going to analyze all these stages: how you are going to analyze the G1 phase, S phase, G2 phase, and M phase? Right. You can actually use a DNA binding dye. Remember that the amount of DNA present throughout the cell cycle is going to vary, and that can be mapped with the help of the DNA-binding dye. So one of the classical DNA-binding dyes is called propidium iodide.

It is a fluorescent dye, and this is the structure of propidium iodide. What you see here is actually having the positive ion attached to the dye, and it is also going to have two iodine molecules; it has been used for the analysis of the cell cycle to label the DNA. So what you do is label the DNA within the cell, and the cell is actually going to have different amounts of DNA during the G1 to M phase. PI has the unique property of being unable to enter live cells but capable of permeating the cell membrane of deceased or dying cells. Through its binding to the DNA and subsequent emission of red fluorescence upon excitation with a specific wavelength, PI enables the evaluation of individual cells' DNA content and facilitates the examination of their distribution within the cell cycle.

What are the materials you require for performing the cell cycle analysis by the PI? So you require the 6-well plate, propidium iodide, RNase A, PBS, alcohol, flow cytometer, centrifuge, 15 ml falcon tubes, 1.5 ml centrifuge tubes, trypsin EDTA, and the 37ºC incubator. How are you going to do the cell cycle analysis? So what you do is seed 10^6 cells per well in a six-well dish 12 hours prior to the experiment. So this means you can probably do the seeding of the cells at night so that in the morning you can come and do the treatment. On the day of the experiment, wash the cells twice with PBS, treat the cells with the desired concentration, and incubate for 24 hours.

This timing is not very strict. You can do 12 hours, you can do 18 hours, you can do 24 hours, you can do whatever time you want, right? It depends on the treatments and other kinds of parameters. After 24 hours of treatment, remove the media and scrape the cells with trypsin or 0.6% EDTA, depending on what you want to do. So if you use trypsin, the cells will actually come off by cleaving the cell adhesion receptors.

But if you use the 0.6% EDTA, it will not do that. So, depending on your overall objective, you can use either trypsin or EDTA. Then you centrifuge at low RPM and discard the supernatant so that you can collect the cells for your analysis. Then you stain these cells so you can keep the cells in 250 microliters of PBS and keep the suspension on ice for a couple of minutes. Then you add 1 ml of 70% ethanol to fix the cells.

Finally, you transfer the cells into a -20 degree freezer for 3 to 4 hours. After incubation with 70% ethanol, centrifuge the sample at 5000 rpm for 10 minutes, discard the supernatant, then add 1 ml of ice-cold PBS. Centrifuge the sample at 5000 rpm, and after

the RNA degradation by the RNase, stain the cells with PI. The final working concentration is 50 to 70 micrograms for 30 minutes before acquiring the data in a flow cytometer. After that, you are going to data acquisition.

So you are going to use the flow cytometer. And so, after staining, analyze the cells under a flow cytometer equipped with the appropriate filter for propidium iodide. So propidium iodide has an excitation wavelength of 488 nanometers and an emission wavelength of 585 nanometers, which means you can probably use the appropriate excitation and emission filters and adjust the flow cytometer settings for appropriate fluorescence and forward and side scattering parameters. So this is all we are not going to discuss in this particular course. But if you are interested, we have discussed these instruments in another MOOC course, which is called Experimental Biotechnology.

So where can you actually get more details about how we can use flow cytometry? So you can actually go through with that lecture. Right. It's called experimental biotechnology. Run the stained sample on the flow cytometer and collect the data for at least 10,000 events. Analyze the acquired data using the flow cytometry software to plot the DNA content, which will represent the signal from the propidium iodide.

With cell count on the y-axis, the resulting histogram will typically show the distinct peaks representing the different phases of the cell cycle, which include the G1 phase, S phase, and G2/M phase. And then you can analyze the data using the analysis software, which is called the FCS-5 Express software. While you're doing these experiments, you are supposed to take a lot of precautions. To avoid any disruption in the distributional cell population, it is recommended not to seed or treat the mammalian cells when they reach a confluency level of 90% to 100%, as this means that if the cells are very old, they may not actually respond or show the distribution of the different cell stages. This is because, at this stage, the majority of cells are in the G1 phase, and using the cells that have attained 100% confluency can impact the overall distribution of the cellular population.

To avoid obtaining unwanted background signals, it is important to run the cell labeled with the proprietary iodide in the PBS when using the flow cytometer instead of using the complete or incomplete medium. And then you need to ensure that excessive pipetting when scraping the cells from the plate is avoided because that is also going to damage the cells, right? So we have prepared a small demo so that you can understand how to use flow cytometry and how to use propidium iodide to study cell cycle analysis. Hello everyone, in this video we will be discussing how to perform cell cycle analysis on FACS equipment. Basically, cell cycle analysis gives us details about how much of the population of a given number of cells is present in each phase of the cell cycle, for example, the G0 or G1 phase, the synthesis phase, or the G2 or M phase. We can design the experiment according to our needs.

For example, if you want to find out how a chemical compound inhibits certain populations of cells, we can do time-dependent experiments, or we can also do a concentration-dependent experiment. So, in both ways, we can find out which particular phase of the cycle is being inhibited. The procedure for cell cycle analysis is very simple.

First, we trypsinize the cells from a 100 mm cell culture dish and seed approximately 1 million cells in a 6-well plate. After the cells are seeded, we incubate them for 12 to 14 hours so that the cells adhere completely to the dish.

After the cells are adhered, we give the appropriate treatment after washing them twice with PBS. So, after the appropriate amount of treatment, let's say 12 hours, 24 hours, or 48 hours, we trypsinize the cells and collect the pellet. We wash the pellet two times with PBS, then centrifuge again and resuspend the pellet in 1 ml of 70% ethanol. This mixture is kept at -30 degrees for 12 hours to fixate the cells. After the cells are fixed with 70% ethanol, we wash the cells with 5 ml of PBS and then collect the pellet and resuspend it in 1 ml of PBS.

Then we provide the appropriate RNA treatment to remove all the RNA from the mixture because RNA, if present in the mixture, might interfere with our cell cycle analysis. After incubating the sample with RNAs for two hours, we give the appropriate propidium iodide treatment. The working concentration for propidium iodide in cell second analysis is 50 micrograms per ml. After giving the treatment with propidium iodide for around one hour, we analyze the cells using FACS equipment. After the samples are prepared, we need to analyze the data in the SPACs equipment.

The first thing we do is open the CellQuest Pro software. After that, we connect it to the cytometer. After connecting to the cytometer, we need a few things such as counters, detector and amps status, and we also need the dot blot press acquisition and analysis. We need another dot blot for FL2A and SSC because in the FL2A channel the propidium iodide emits red fluorescence. We also need a histogram to see the cell cycle phases; for that purpose, we need the FL2A channel.

We need another dot blot in order to see whether there is a presence of any doublets, and there is a chance that there might be a presence of doublets in the data, like, for example, the clumps of cells, so we have to exclude that from the data. For that purpose, we take the FL2 and FL2W channels. Now that everything is ready, we can change the directory for acquiring the data, and we can also choose the file name. So we'll name it as "untreated one," and we'll keep the file count to one for checking the data we have kept on the setup so that we can see whether the data is coming in properly or not. Then we can remove it from the setup and acquire the data.

So now we are going to load the sample onto the sample injection port and then press acquire. As we can see, the number of events that have started recording in the FSC and SSC plot shows that most of the population is seen between the 200 and 300 mark. The population that is moving away from the doublets in FL2A and SSC indicates that there are three different populations: G1, S, and G2. In the fl2a and the counts, you can see the longest peak is the G1, the one in between is the S, and the smallest peak is the G2. Before acquiring the data, we have to set the number of events, so we go to acquisition and storage and press record 10,000 events.

In order to record the data, we have to remove it from the setup, then set it up, and then

press acquire. In the FL2 and the FL2W channel, we can see that there are two different populations: one is a thin line, and the other is the presence of some doublets or clumps of cells. This population might be the presence of the doublet cells or clumps of cells, so while acquiring the data, we can see this. However, in the FCSXpress software, we can remove that.

Now that we have acquired the data, we can proceed. We can take the data from the untreated sample; we have to remember that we don't have to change the parameter conditions in order to compare the untreated and treated samples. Now we have to change the sample name from untreated to treated and also change the file count to one. It's okay, and then acquire. We can see that there is a little shift in the S and the G2 phase, but we can safely say for sure that there is a change in the data. For that, we have to process the data on the FCS Express software in order to see what the difference is between the untreated and the treated sample.

So now we will remove it from the setup and then acquire the data for the treated sample. Sometimes we can see that the events per second will be low. So, in order to increase the events per second, we can pause the recording and then tap the sample once or twice in a while to shake the sample, and then the flow will be continuous again from the sample injection port, and the events per second will increase. Now we can see that the events per second have increased because we have tapped the sample and the data acquisition will be a little bit faster. So now we have acquired the data for the untreated and treated samples in order to see in which phase of the cell cycle the arrest has taken place, so we have to process the data in the FCS Express 5 software.

Now that we have acquired the data on the FACS equipment, we have to process the data in order to see the difference between the untreated and treated samples; for that purpose, we use the FCS Express flow file software to process the data. So for that purpose, we use the new layout and then change the mode to landscape because it is easier to work in landscape. After that, we go to the data in the toolbar, press open, and then go to the folder where our data is saved and press open the untreated file, as we can see that there are multiple options available: dot density, color, dot contour, surface, and histogram. And multi-cycle DNA and kinetics for this cell cycle DNA, we only need the multi-cycle DNA plot and the dot plot, so we are going to open these two. Because we have recorded our data on the FL2A channel, and also because the propidium iodide is only shown in the FL2A channel, we are going to open the FL2A channel.

So now, after we have opened the dot plot and the cell cycle DNA plot, we can see how much of the population of cells is represented by the G1, S, and G2 phases by right-clicking on the plot and then selecting statistics and then show DNA cycle statistics. So a small window will appear in which it will show the percentage G1, the percentage G2, and the percentage S. This is for the untreated sample. Let's see the treated sample. As we can see, there is some change in the untreated and treated samples, both in the dot plot and in the cell cycle plot.

To see how much percentage of the cell cycle phases have changed, we right-click on the

plot, then show DNA cycle statistics, and place it right beside the untreated one for comparison. We can see that there has been a reduction in the G1 phase from 61 to 49. From G2, there has been a decrease from 15 to 12, but there has been an increase in the percentage of the S phase, which has increased from 23 to 38. Therefore, we can say that there is a significant change in the phases of the cell cycle; however, we can only be sure after conducting the experiment in triplicate so that we obtain the proper standard deviation and the standard error.

This is one way of processing the data. Another way of processing data is by getting, as we can see in the FSC and the SSC channel, that there is some debris near the zero-zero point. And also, there are some populations of cells which are very far from the 00 representing clumps of cells or maybe doublets, which we have to take care of using gating techniques. In order to explain the gating, I will use another page, then open the data and press OK. For gating, we need multiple plots; the first one is FSE and SSE, the second one is between FL2A and SSE, and the third one is between. FL2A and FL2W; the fourth one is between FL2A and FL2H, and the last one is between FL2H and FL2W.

So, we have to start the gating from the first plot, the FSC and the SSC. In order to do the gating, we have to go to the gating option in the toolbar, and then we can choose any one, like ellipse, rectangle, polygon, or freeform. These are the shapes of the gate. So we'll go with the polygon gating because it is easier to handle. And then we can obtain our required population using the polygon gating tool and then just select the required population.

So here we are, excluding the debris and the clumps of cells that might be interfering with our actual data. And then we can select the gate color we want to assign and also rename gate one to our particular type, but we'll go with the standard one and then press OK. And then we have to apply this gating to the second plot. We can just drag and drop it onto the second plot.

So we can see here that we have excluded some populations. So, there is still some population that can interfere with our data. So we'll do the gating again and then we'll exclude some more population which might not be helpful to us. And then we'll name this gate 2. So in order to apply this gate to the third plot, just drag it and drop it. And then we can see that another number of the population has reduced, and in the third plot as well, we can reduce this population, which is a little bit distinct from the singlet cells.

So we have taken another gating using a polygon, and then we are only now going to select the singlet cells, and then we will name this as gate 3. From gate 3 onwards, we just have to apply the gates to the next plot. And then we can apply the gate to the plots by just selecting the plot and then going to the top left corner and selecting the gate. So, as we have seen, we have reduced a significant number of the population, and we have only selected the population that might be helpful in processing the data. And then finally we apply gate three, which is the final gate, as you will then see the change in the cell cycle statistic plot.

So this is for the untreated one; in order to show the cell cycle statistics, just press statistics and then show DNA cycle statistics. As we can see in the ungated one, we have seen that the percentage of G1 was 61, whereas in the gated one it is 67.

1. And the percentage G2 is 8.9, and the percentage S is 23. Similarly, we can do the same for the treated one, but we don't have to follow the whole procedure; we can just copy all the plots, go to a new page, press space, go to data, and then select the treated one. It will just replace all the plots with the treated data, but the treated data and the untreated data are a little bit different, so we just have to move the gate. In order to see the cell cycle status for the treated one, we can select the DNA statistics. And then we get the DNA cycle statistics. So in this way, we can process the data for the untreated and treated samples in the cell cycle DNA statistics using both gating and non-gating techniques.

So hopefully this video is helpful for everyone. Once you analyze and get the data, this is the result you are going to get, right? So this is the sample from the untreated samples. This is a cell treated with the anticancer compound, right? So, for example, this is actually the data from the MDA-MB231 breast cancer cells. and they are either not being treated or being treated with the anti-cancer compound. So what you see here is actually the G1, right? So, this is the G1 phase. This is the, you know, the G2 phase, right? And this is actually being called the S phase.

And what you see here is very small, which is actually going to be called the M phase, right? So what you see here is that there are a lot of cells in the G1 phase, and there are also cells in the G2 phase. Then you will see, in terms of percentage, that 54% of the cells are in the G1 phase, 67% are in the G2 phase, and 39.4% are in the S phase. When we treated it with the anti-cancer compound, the cells present in the G1 phase were 72.

The cells present in the G2 are also 6.41. But the cells present in the S phase actually got reduced to 20%. So that means it is actually affecting the cell cycle. Whatever compound you are using, it is actually affecting the distribution of the cells within the cell cycle. So, what is the role of the cell cycle? So we have discussed the cell cycle.

We have discussed the different stages of the cell cycle. But what is the significance of following the cell cycle? So it is required for development and growth. It is required for cell replacement. It is required for regeneration. It is required for asexual reproduction. Talking about development and growth, the transformation of a single cell into a multicellular system is possible due to the cell cycle and cell divisions.

And after the lifespan is over, it has to be replaced. Right. And after that period, it needs to be replaced with a new one. And it is possible due to cell division and the making of more cellular copies. For example, human RBCs have a lifespan of three months or approximately 100 days.

New RBCs are formed in the bone marrow by cell division. Similarly, we have regeneration. Cellular damage and injury are integral parts of the living system. Cell

division is the primary event required for the synthesis of lost or damaged organs. So it could be an injury from inside, or it could be an injury from outside. So when you get a cut, the blood comes out, and then the regeneration process starts. Similarly, you might have seen the regeneration in lizards and other small organisms where they are actually able to regenerate whole organs, and in this process, mitosis is taking place.

Then it is also required for asexual reproduction. So asexual reproduction is common in lower invertebrates. In these organisms, the cells divide to form new cells, and these newly formed cells give rise to the new organism. For example, the Hydra, amoebas, and all those kinds of new organisms. The cell cycle has to be under tight control, and that is what we discussed in lecture number one: how it is different if there is a complete set of protein machinery that recognizes the different stages of the cell cycle, and therefore regulates the cell stages.

Right, so the cell cycle at different steps is tightly regulated by the cell cycle checkpoints. These cell cycle checkpoints are used to ensure the completion of different steps and the repair of cellular damage. The main checkpoints are present at G1/S, G2/M, and M phases, and each point is controlled by the mutual interaction between the cyclins and the CDKs. And we have discussed all of these in detail regarding how the regulation is happening. And P53 and RB proteins are also involved in the control of the cell cycle stages G1 and G/M. Now the question is what will happen if these events go wrong, as dysregulation of the cell cycle and control mechanisms give rise to tumors.

After a certain number of cell divisions, all cells enter the G0 phase and cease cell division. In the case of tumors, cells lose control and multiply indefinitely to give rise to the cell mass, and these cells take in nutrition but do not perform the functions necessary; thus, retinoblastoma, like the RB proteins and p53, are crucial cellular factors responsible for cell cycle control and play a crucial role in tumor development. So we have discussed in detail the cell cycle, cell division, growth media, the different stages of the cell cycle, and so on. If you are interested in studying more and want to learn more about these events, then you can actually follow this particular research article, which is very good at explaining the role of the checkpoints, cycling, and the CDKs. So, with this small discussion about the cell cycle and how you can study the cell cycle in the mammalian system, I would like to conclude my lecture here. In our subsequent lecture, we will discuss some more aspects related to cell division and the cell cycle. Thank you.