Experimental Nanobiotechnology

Prof. P. Gopinath

Department of Biosciences and Bioengineering,

Indian Institute of Technology Roorkee

Lecture 18: In vitro Cytotoxicity Analysis

Hello everyone, today we are going to learn in vitro cytotoxicity analysis. In today's lecture, we will be learning in vitro cytotoxicity analysis and classification of in vitro cytotoxicity analysis. We will be also learning trypan blue exclusion and MTT assay, both theoretically as well as practically in today's lecture. Let us see what is in vitro cytotoxicity analysis. Here, we will be using the cell-based assay to screen collections of compounds to check whether the test molecules or nanomaterials are cytotoxic or biocompatible in nature.

Regardless of what type of cell-based assay we are using, it is important to determine how many viable cells remain at the end of the experiment. And the selection of assay is one of the important parameters. We have to select the right assay and we have to select the right cell lines according to our material. What are the advantages of this in vitro cytotoxicity analysis? It is fast and cost effective and it is reliable and reproducible.

And also, this will be useful for testing large and different sample at the same time. Also, more prediction probability is possible here. Based on endpoint measurements, these in vitro cytotoxic analysis are classified into different categories. The first one is dye exclusion method. This method is based on exclusion of dye by the live cells.

and here the dead cells have ruptured plasma membrane due to which the dye will penetrate inside and stain the dead cells that means the live cells don't take up the stain whereas the dead cells will take up the stain and the dead cells will appear blue color if you are using trypan blue the dead cells will appear blue color the next assay is Calorimetric assay. This technique is measurement of biochemical marker to quantify cellular metabolic activity. When the reagents come into contact with viable cells, they produce a color reaction and the color can be quantified using a spectrophotometer.

Some of the example for this assay is MTT and MTS assay. What happens is if the more cells are viable, they will be more metabolically active. And this will convert the particular

chemical into more color product. If you are getting more color, it implies that the cells are more viable. More viable cells means color intensity will be high.

The next assay is fluorometric assay. This fluorometric assay is an alternative to dye exclusion and colorimetric methods. This fluorometric cell viability method is based on non-specific cleavage of non-fluorescent chemicals such as fluorescein dye acetate and which will give fluorescence when cleaved by cellular esterase. When this dye enters into the cell, inside the cell, The cellular esterase will break this fluorescent diacetate and it will produce fluorescence. The fluorescence signal can be measured and that can give the number of viable cells.

If we have more viable cells, that means more fluorescence. The next assay is the luminometric assay. This is similar to the fluorometric assay, but here we will be measuring bioluminescence. This bioluminescent test is based on the correlation between a bioluminescent reaction and the impact of the tested substance. The impact might be increased cell proliferation or cell death. Here, we will be using this luminometer to measure the bioluminescence.

Let us see the trypan blue exclusion and MTT assay in more detail. The first one is the trypan blue exclusion assay. Trypan blue is a large, negatively charged molecule, and it is used to determine the number of viable or dead cells in a cell suspension. The principle of this assay is that live cells have intact cell membranes, which exclude the dye. As I mentioned earlier, live cells have intact cell membranes, so they won't take up the dye.

Whereas in the case of dead cells, it has a ruptured cell membrane. It will take up the dye and the cells will become blue color. This assay can be useful for both adherent and non-adherent cells. We can treat the cells with serial dilutions of the test substance. For example, if we are using nanomaterials, we can use different concentrations of nanoparticles and incubate the cells for different durations. It can be for 24 hours, 48 hours, or 72 hours, depending on your material's toxicity or the experiment's requirements.

At the end of the experiment, you have to wash the cells and suspend them in the appropriate medium. Then, the dye will be added to the cell suspension. Once you add the dye, the viable cells will have a clear cytoplasm, whereas the dead cells will have blue-colored cytoplasm. The number of viable or dead cells can be calculated by counting the number of blue-colored cells with the help of a hemocytometer, or you can use an automated cell counter. Let us see the overview of the trypan blue exclusion assay. Once the cells are 70% confluent in the tissue culture flask,

Remove the media and wash the cells with DPBS, which is Dulbecco's phosphate-buffered saline. Then, add the dissociating agent, which is 0.25% trypsin-EDTA. Then, incubate the cells in a 5% CO_2 incubator until complete detachment of cells. You can check the cells under the microscope to confirm that all the cells are detached. Once the cells are detached, add media to the volume of 2x the dissociating agent.

Then, transfer it to a centrifuge tube and centrifuge the cells. Once the cells are centrifuged, the pellet contains the cells, and the supernatant contains the medium as well as the dissociating agent. We have to discard the supernatant very carefully without disturbing the cell pellet. Then, the cell pellet can be resuspended in media. Then, we have to add 0.4% trypan blue staining solution in a ratio of 1:1. Once it is mixed, add the stained solution to a hemocytometer, and the cells can be counted under the microscope.

Or if we have an automated cell counter, we can take the cell counting chamber slide. Then add the stain solution and insert the slide into the automated cell counter. It will count the number of cells and provide complete data on how many cells are dead and how many are alive. We have to calculate the percentage of cell viability by dividing the number of viable cells by the total number of cells and multiplying by 100 which is mentioned below.

$$\text{Cell Viability \%} = \frac{\text{Number of viable cells (unstained)}}{\text{Total number of cells}} \times 100$$

This allows us to calculate the number of live cells.

The next assay for understanding cell viability is the MTT assay. The MTT assay is one of the most widely used colorimetric assays for assessing cytotoxicity or cell viability. This technique is mainly based on the mitochondrial enzyme succinate dehydrogenase. This enzyme converts the water-soluble yellow dye MTT into insoluble purple formazan crystals. These can be dissolved, and absorbance can be measured at 570 nanometers to calculate cell viability.

Higher color intensity means more viable cells are present. Let us see an overview of the MTT assay. We have to add the cells with media into the plate in an appropriate count. We must count and add a suitable number of cells into a 96-well plate. Once the cells are added, incubate them for 24 hours in a 5% CO_2 incubator.

Once the cells are nicely attached and grown, you have to add the test material. The test material can be nanoparticle or it can be a biomaterial or any small molecules or

compounds. Once you add the test material, incubate the cells along with the test material for 24 hours or more than 24 hours according to the requirement of the experiment. Then remove the media carefully and add MTT dye. Then again incubate for 4 hours in 5% CO_2 incubator

Then remove the MTT dye. Then add the lysis buffer to dissolve the formazan crystal. Incubate until complete dissolution of formazan crystals. Once it is completely dissolved, then record the reading using plate reader. And the observance can be measured at 570 nanometer. As I told earlier, if you are having high color intensity, that implies that more number of cells are viable.

Tabulate the data and the cell viability can be calculated by using the formula A test by A control multiplied by 100 here this A denotes absorbance then we can analyze the data which is mentioned below.

$$\text{Cell Viability \%} = \frac{A_{test}}{A_{control}} \times 100$$

for example in this case the control which is 100 percentage and the sample one we can see the cell viability is more than 80 percent that means the sample one is biocompatible material if the cell viability is more than 80 percent the material is biocompatible in nature whereas you can see that sample 3 the cell viability is less which is toxic in nature

So based on this, we can easily understand the cytotoxicity of your material. Let us see the troubleshooting for the MTT assay. The first problem is the MTT reagent is blue-green. It may be due to contamination with a reducing agent or bacterial contamination. Discard it and use a new MTT aliquot. Or it may be due to excessive exposure to light. These are light-sensitive, so you must store them in the dark at 4 degrees Celsius.

The next problem is replicates having different values. Whenever we do experiments, we must always do triplicates. If you are having different values, it may be due to inaccurate plating or pipetting. For example, in the 96-well plate, when adding the cells, the cell number should be uniform in all wells. If there is a pipetting error or a plating error, that will give an inaccurate result. That's why, to overcome this, we must use the right number of cells when plating.

We must avoid pipetting errors and ensure the pipette is working properly. The third problem is absorbance readings being too high. It may be due to the cell number per well being too high. If the cell number is higher in the wells, the absorbance will be very high. Then you must decrease the cell density during plating.

Or it may be due to the contamination of culture with bacteria. The bacteria grows very rapidly. So if there is a bacterial contamination, that will also lead to increase in the absorbance reading in this case we have to discard it and we have to use the fresh mtt reagent and we have to repeat the experiment and the last problem is the blank if the only medium also is giving absorbance reading okay in that case the medium is contaminated with bacteria or this medium contains ascorbic acid if it is contaminated with bacteria we have to discard it and

If the medium is having ascorbic acid or if you are not incubating this MTT assay in the dark, so that will lead to increase the absorbance in the blank also. So always incubate the plate in the dark or we have to use the suitable medium, depends on your cell type. And next problem is absorbance readings are too low. So it may be due to the cell number per well is too low. or the incubation time for reduction of mtt is too short in that case you can increase the incubation time until the purple color is developed

if the cell number is too low we can increase the cell density while plating and it may be due to incubation time for solubilization of formazan dye may be too short in this case we can increase the incubation time with the detergent reagent until all the formazan crystals are dissolved and this may be also due to cells are not proliferating due to improper culture conditions or inadequate time to recovery after plating that's what i told you in the mtt also first step once you add the cells to these 96 will plate we have to allow the cells to attach and grow at least for 24 hours once the cells are nicely attached and grow then only we have to add the test compound So check the culture conditions and medium temperature and humidity and make sure that the cell condition is proper before we add the test compound.

Let us go to the lab and learn this in more detail. To perform the cell culture experiment, We first need to UV-sterilize the biosafety cabinet. Simultaneously, preheat the required materials to 37 degrees Celsius. Once UV sterilization and preheating are complete, we will begin the cell culture experiment.

For performing the experiment, we require DPBS, which is Dulbecco’s phosphate-buffered saline. 0.25% Trypsin EDTA, complete cell culture medium, 96-well plates, centrifuge

tubes, microtips, 3 sets of micropipettes, microcentrifuge tubes, and a glass beaker to discard the waste. Next, we will observe the T25 flask under the inverted microscope to check the cell confluency. If the cells are about 70% confluent, we can proceed with the experiment. Take out the flask from the CO_2 incubator.

Now, we will examine it under the inverted microscope to confirm the confluency of the cells. Here, you can see the cells are spread throughout the T25 flask, so we will proceed further. The first step is to discard the media and wash the flask twice with DPBS. After the washing step, we will add 500 microliters of the dissociating agent, which is 0.25% trypsin EDTA, and gently spread it throughout the flask. Now, we will incubate the flask for 5 minutes in a 5% CO_2 incubator.

We will then observe the cells under the inverted microscope. As seen here, the cells have detached from the flask, and we can now proceed to the centrifugation process. Next, we will add twice the volume of complete media to that of trypsin to the flask and slowly spread the media throughout the flask. Then, transfer the entire mixture into a microcentrifuge tube. Add an equal amount of media to a blank microcentrifuge tube.

Now, centrifuge both microcentrifuge tubes. After centrifugation, we can observe the pellet formed at the bottom of the tube. We have to carefully discard the trypsin-containing media without disturbing the pellet. Next, we will add fresh media to the microcentrifuge tube. Using the media, slowly resuspend and dispense the pellet.

Now, we will add an equal volume of 0.4% trypan blue to the media containing cells and mix well. Next, add 10 µL of the mixture onto both sides of the cell-counting chamber slide. Switch on the automated cell counter, place the slide into the machine for cell counting, and press 'Count Cells.' Once the count is complete, save the data to an external disk and open it on the monitor to view the exact cell count. So, from this data, you can clearly understand the percentage of live cells and dead cells.

Here the live cells are represented in the green and dead cells are represented in the red color by the software. So here the live cells are 95% and the dead cells are 5%. Here you can see there are approximately 67 lakhs live cells present per mL. For the MTT assay and apoptotic studies, We need a concentration of 10,000 cells per 100 microliter.

So for this we will add 150 microliter of the cell suspension to 9.85 mL of complete media which will give us the required cell concentration. First add 150 microliter of the media containing cells to the centrifuge tube. Then add 850 µL of media followed by 9 mL of

media. Once we have prepared the exact cell concentration, we will seed the cells in a 96-well plate. One plate for the MTT assay and another one for the apoptotic studies.

For the MTT assay, we need 6 wells, 3 for the control group and 3 for the test group. For the apoptotic study, we will perform 2 assays, AO EB and Hoechst staining. So for this, we require 12 wells in total. Now we will add 100 microliter media containing 10,000 cells to each of the 18 wells for both experiments. First we will seed the cells for the MTT Assay followed by we will seed the cells for the apoptotic study.

Here, we have added the cells into a 96-well plate. Now, we will observe the cells under the microscope. Then, incubate the plate in a 5% CO_2 incubator to proceed with the experiments. We have seeded 10,000 cells for the MTT assay. Now, we are going to view them under the inverted microscope to confirm

whether the cells are present in both the control well and the well where the test material will be added. After confirmation, we will place the 96-well plate in a 5% CO_2 incubator for 24 hours. Yesterday, we seeded the cells for the assay. Today, we will prepare the desired concentration of the test material for treatment. The concentration we prepare must be diluted in the cell culture media.

Once it is done, we will take the seeded cells from the incubator and treat them with the test material. Here, we can see that the cells are well adhered to the 96-well plate. After confirming the attachment of the cells, we will treat them with the test material. For the control group, we will add 100 µL of the cell culture medium. While in the remaining 3 wells, we will add 100 µL of the desired concentration of the test material.

We have to incubate it according to our treatment period. Here we will incubate it for 24 hours and we will observe the results after this incubation period. After the incubation, we have to carefully remove the medium from the wells and add 100 microliter MTT dye. Since MTT dye is sensitive towards light, it should be added in a dark environment. Once we add MTT dye, we will incubate the plate for 3 to 4 hours.

After incubation, we can observe the formation of formazan crystals in the control wells, while the test wells show fewer formazan crystals compared to the control. Next, we will carefully discard the MTT dye and add 100 µL of lysis buffer to each well to dissolve the formazan crystals. The plate will be incubated at 37 degrees Celsius for 10 minutes. After incubation, we observe the violet coloured formozan crystals. Finally, we will measure the absorbance at 570 nanometer and plot a graph based on its absorbance value.

Here we have used IC50 concentration of a drug and studied its efficiency against the MG63 cell line. So that is the bone cancer cell line. And here you can observe the cell viability has gone down approximately 50%. So this indicates the IC50 concentration. So that means the concentration required to inhibit 50% of the cell growth. As a summary, in today's lecture, we learnt about in vitro cytotoxicity analysis and its classification. We also learnt trypan-blue staining and MTT assay both theoretically as well as practically in detail.

Thank you all for your kind attention. I will see you in another interesting lecture.