The Basics of Wet Lab Work in a Bioresolution Lab

BSE399A (Undergraduate Project) - END TERM REPORT

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INTRODUCTION

In Biological Sciences, to make any discovery, generally, the plan of action begins with proposing a theory and following it up with experiments to verify it. In the case of cells, or any biological system for that matter of fact, things are usually just too small to observe with the naked eye for verification of the proposed theory. Most of the time, to observe things at such a small scale (of the order of a few micrometers), microscopy is used. With time and the advancement of technology, just like other things, microscopes have also developed vastly, and have become specialized for different kinds of specimens.

I am a third-year undergrad student at IIT Kanpur, and due to the Covid-19 pandemic, I and my batchmates in the department of BSBE could not attend any labs from our department during our 2nd year and missed a major component of our training as undergraduates of the department. Thus, I decided to undo that for myself by opting for this UGP course and attempting to gain basic lab experience, under Professor Nitin Mohan.

In the UGP, I got to know (and perform myself) the basics of Immunostaining, Cell Viability Assay (MTT Assay), and TIRF microscopy.

THE THREE EXPERIMENTS

Immunostaining

Immunostaining is the process of staining cells or certain parts of them by the combined use of two types of antibodies- primary and secondary. The primary antibody is specific to the part of the cell one wants to observe, while the secondary antibody has the ability to fluoresce (when illuminated by radiation of a specific wavelength) and binds to the primary antibody.

I performed immunostaining of BSC-1 cells for acetylated and detyrosinated microtubules and observed their staining under the microscope. The primary and secondary antibodies used are shown in table 1.

Type	Degree	Name
detyrosinated	1 (primary)	Rabbit anti-detyrosinated
acetylated	1 (primary)	Mouse anti-acetylated
detyrosinated	2 (secondary)	Donkey anti-rabbit
acetylated	2 (secondary)	Donkey anti-mouse

Table 1: The set of primary and secondary antibodies used in the immunostaining experiment

TIRF Microscopy

TIRF (Total Internal Reflection Fluorescence) microscopy was the technique used by me to image the immunostained BSC-1 cells. This technique is used to image cells when the part to be observed lies just near the membrane of the cells. TIRF microscopy employs Total Internal Reflection of light when it goes from a denser medium to a rarer medium, to illuminate only the parts of the cell in close proximity to the boundary between the denser and rarer media (an evanescent wave is released at the boundary, which decays exponentially in the rarer medium). In the case of microtubules, since they are part of the cell's cytoskeleton, they belong to this zone of close proximity to the membrane, and thus TIRF microscopy is ideal for their imaging.

TIRF Microscopes come in two categories- P-type (prism type) and O-type (objective type). In our lab, there is the O-type microscope, which works in the inverted configuration, and requires a better lens generally and

thus is more expensive. However, they also provide better flexibility of the working sample.

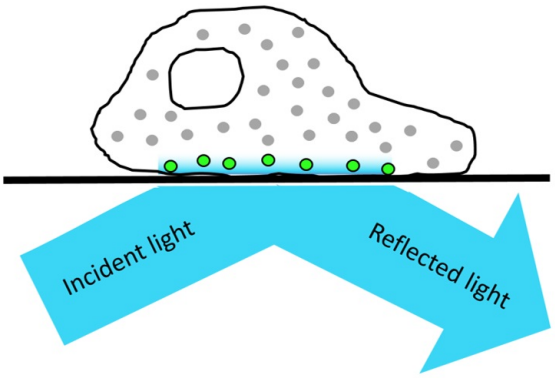

Fig. 1: Excitation of membrane-near parts of a cell in TIRF Microscopy

Cell Viability Assay (MTT Assay)

The purpose of this experiment was to analyze the cytotoxicity of various nanoparticles in cells and see how they affect cell viability. Nanoparticles have many applications in biology such as in drug delivery, nanoparticles can assist in much more specific drug delivery, by encapsulating the drug particles and targetting them to the right cell type. Thus, information on their cell viability is important to know before they can be used for any such applications to prevent unexpected side effects. We used zinc oxide nanobodies in the experiment.

METHODS

Immunostaining

Materials- 2 coverslips with ~ 36000 BSC-1 cells per slip, PFAGA (Fixating agent, both PFA and GA used as they work together better than either alone), PBS (Phosphate Buffered Saline, used to wash/rinse cells), NaBH4 solution (quenching agent, 1mg/ml), Blocking Buffer (BSA, 3% +TX100, 0.2%), antibodies (as in table 1), Washing Buffer (BSA, 0.2% +TX100, 0.2%)

Aim- To see how well the BSC-1 cells get stained for acetylated and detyrosinated tubulin.

Protocol- i) Firstly take the cells (on coverslip) and fix them using the PFAGA fixator (first PFAGA needs to be given a water bath for like 10 mins after being taken out from the refrigerator). Leave for 10 mins for them to get fixated.
ii) After 10 mins, remove the PFAGA, and rinse with PBS twice. Then, put freshly prepared NaBH4 solution on the coverslip/s and wait for 7 minutes. After 7 mins, rinse with PBS twice.
iii) Now, we need to add Blocking Buffer. After rinsing with PBS twice in the previous step, add blocking buffer to the cells. The blocking process will take one hour.
iv) Next, we have to add primary antibodies to the cells. We first make a mixture of antibodies. We take 1 microlitre from the microtube where it is stored and add it into 99uL of blocking buffer.
v) Put a small drop of the mixture made in the previous step on a glass slide and put on it the coverslip with cells so that the cells face the drop of antibody mixture. This is a very carefully done step as the coverslip can break easily. Wait for 1 hour after this.
vi) After 1 hour, transfer the coverslip back to the hexaplate where coverslips were initially present. Rinse once with washing buffer. Then wash (leave for 5 mins) with washing buffer twice.
vii) Then, again make secondary antibody mixtures in the same way as the primary ones. We have secondary antibodies in the lab itself; we have attached dyes to them in-house: as we need two types of dyes, being a STORM lab (activator dye-405nm and reporter dye- 647nm). Put a drop of the mixture on a glass slide. Transfer the coverslips on the glass slide, again taking care of the side where the cells are. Leave for 50mins. NOTE: switch off all nearby lights while working with secondary antibodies as we don't want them to get excited by light right now.
viii) Rinse with washing buffer followed by washing thrice.
ix) Mount the cells on a glass slide, to be observed later under the microscope.

Cell Viability Assay (MTT assay)

Materials- 96 well plate with ~ 10000 HeLa cells per well, 1mg/ml solution for each compound, autoclaved MilliQ water, bath sonicator, media composed of DMEM (Dulbecco's Modified Eagle Medium) + 10%FBS (Fetal Bovine Serum) + 0.1%antibiotic (to reduce contamination), DMSO (Dimethylsulfoxide for dissolving the formazan crystals post-MTT solution treatment), MTT solution (5mg/ml in Milli-Q water), Zinc Oxide nanoparticles (which are covered with a stabilizing interfacial layer of organic nature)

Aim- To measure the cytotoxicity of different nanoparticles, using the MTT assay.

Protocol- i) Firstly, the HeLa cells were grown in the cell culture overnight, in a 96 well plate, in the media (200uL per well), not containing nanoparticles. In parallel, 1mg/ml solutions of the nanoparticles are made in milli-Q water. By using the C1V1=C2V2 formula, the following concentrations of nanoparticle solutions were made (in ug/mL): 5, 10, 50, 100, 200, 400, 600.
ii) After overnight growth of the cells, the original media was replaced by nanoparticles containing media, in the same volume (200uL per well).
iii) Then, the resultant mixture was incubated for 72 hours in an incubator at 37deg C.
iv) Then, to every well, 20uL MTT solution was added from the stock solution (5mg/ml), without removing the media already in the wells.

v) Then the plate was incubated in the incubator for 3 hours, at 37deg C and 5% CO2 to maintain optimum temperature and pH.
vi) After 3 hours, the plate was jerked over the sink carefully to remove the MTT.
vii) Then, 200uL DMSO was added per well quickly, to dissolve the formazan crystals formed.
viii) Then, the plate was read by a spectrophotometer plate reader (absorbance measurement device), at 572 nm.

RESULTS

In the combined results of the immunostaining and TIRF microscopy, I observed the acetylated (more networks visible due to more of them) and detyrosinated microtubules of BSC-1 cells immunostained by me under the microscope (Fig. 1, Fig. 2).

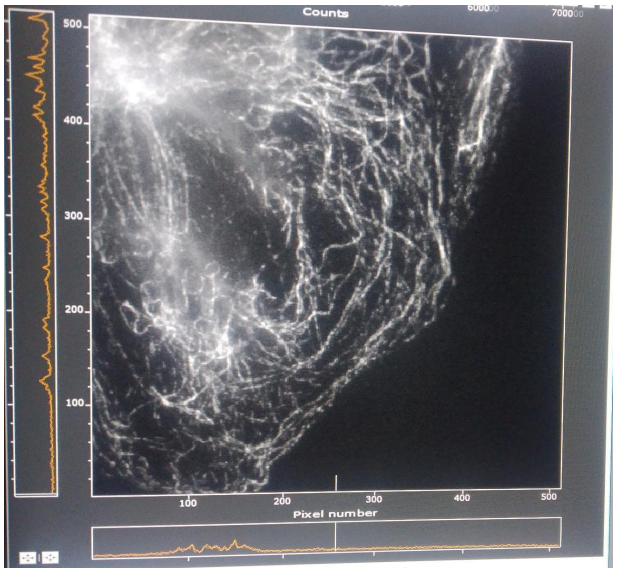

Fig. 2: Acetylated microtubules in BSC-1 cells.

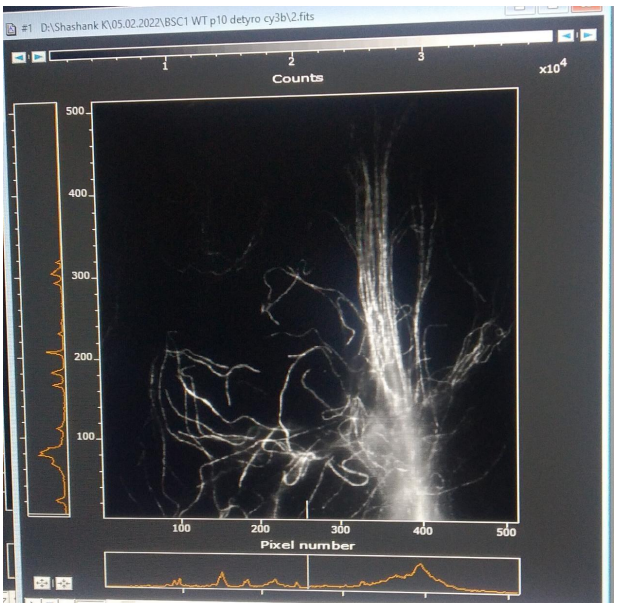

Fig. 3: Detyrosinated microtubules in BSC-1 cells.

The mean cell viabilities according to the observed absorbances for the wells in the cell viability experiment were plotted as lines graphs (Fig. 4). The concentration at which the curves are going below 50% viability are the IC50 (half maximal inhibitory concentration) for the respective compounds. In the figure, 2z is zinc oxide, 1cz has one part casein, 2cz has two parts, and 3cz has three parts.

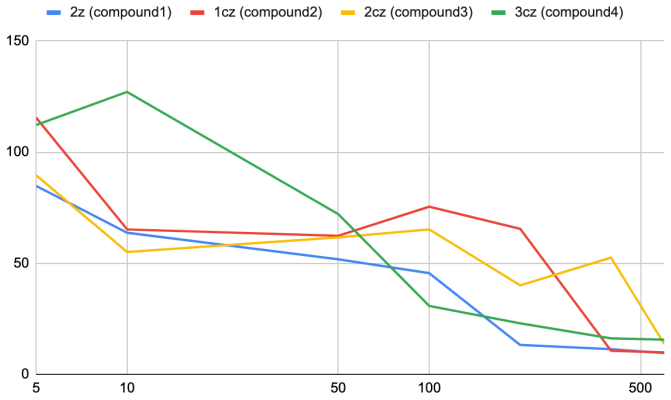

Fig. 4: Decreasing cell viability with increasing concentration of nanoparticles.

CONCLUSION

In conclusion, I performed the above three experiments in-depth, and got the basic results as expected; observing the acetylated and detyrosinated microtubules of cells immunostained by me was one of the highest points of the UGP for me.

Overall, in the two months I worked for this UGP, I learned a lot of things given that I had literally no physical wet lab experience prior to that. Along with performing the experiments described above in full, I also learned some basic skills and practices of working in a wet lab, like using an autoclave machine, cleaning the hood for bacterial culture, weighing substances on the analytical balance, and sonification of substances, etc.

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