View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by IntechOpen

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

4,800
Open access books available

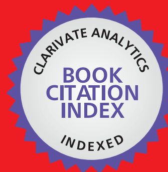
122,000
International authors and editors

135M
Downloads

154
Countries delivered to

Our authors are among the
TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Chapter 5

In Vitro Toxicity Testing of Nanomaterials

Ansie Martin and Angshuman Sarkar

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.80818

Abstract

Cell culture-based techniques are employed world-wide to assess risks to optimize design and handling of materials. Nano-scale particles particularly pose a challenge to the predictability of toxicity. This is due to the interplay of several factors including size, shape, surface properties, micro-environment, charge, electronic species etc., that affect the degree of cellular stress. Numerous assays could be employed to understand the nature of toxicity. Some of these include cytotoxicity, metabolic kinetics, modulation in cellular morphology. Various models such as primary cell culture and cell lines can also be employed that best suits a study. Signaling cascades could also be monitored in better understanding cellular responses holistically. Fluorescence microscopy can further be attempted in studying spatial and temporal variations of bio-markers. Ultimately any plethora of dose-response data, need to be streamlined for developing toxicity evaluation protocols that are accurate, cost effective and time saving.

Keywords: nanoparticles, toxicity, signaling, cell culture, *in vitro*

1. Introduction

Nanoparticles are particles that have at least one dimension less than a 100 nm [1]. Manufactured nanoscale particles find themselves in multitudes of applications such as industrial, food, cosmetic, medical, computing and so on. Distinctive properties such as increased surface area and enhanced reactivity largely fuel nanoingenuity and the net result is an exponential raise in the manufacture and use of nanoparticles. As such human exposure has also risen at an alarming rate. Deleterious effects of this exposure include pulmonary distress, hypertension, cardiovascular damage, irritation of the otolaryngological tissue, impairment of reproductive functions, neural injuries and blood disorders. Long term exposure can also lead to epigenetic alterations and cancer.

IntechOpen

© 2018 The Author(s). Licensee IntechOpen. This chapter is distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Benefits of nanoscale materials are numerous and the market for nanotechnology is enormous owing to the large turnout for profits. The only ethical way forward to manage the harmful effects of nanoparticles is in educated design with innovations, applications and in ethical disposal of nanowastes [2]. Understanding the mechanisms of toxicity attributed by nanoparticles is crucial to this endeavor. Some tools to assess dose and time dependent toxicity have already been developed. Toxicity can either be directly- cell death or the impairment of normal functions in the cell. There are different ways to test toxicity as there are different routes of exposure to the toxic substance.

Conventionally 'acute toxicity testing' has been carried out on a model organism, with each candidate, being tested for a single dose and a single exposure time. Following the exposure, biochemical and histological changes observed from different tissue samples of the dead animal were documented for analysis. The determination of LD_{50}; administration dose with 50% lethality thus required the sacrifice of many animals. LD_{50} has long been used the comparative standard in assessing the degree of toxicity. Several skin sensitivity tests and lymph analysis have also been performed to assess immunogenic potential of an exposing agent. There are other function-based toxicity tests to study effects on reproduction, mutagenic potential, neural management and embryonic development.

The advantage of cell-based toxicity assays is that large numbers of experiments can be conducted to screen the exponential dose-time combinations of exposure [3]. It is greatly time and cost effective as compared to *in vivo* testing. And ethical concerns of animal sacrifice and need for elaborate and regulated laboratories are avoided. No doubt, *in vitro* and *in vivo* results tend to vary depending on the case study, but the wider use of *in vitro* studies allows for only a fraction of the most promising outcomes to be further evaluated with live animal testing. Again, this alleviates many concerns associated with the using animal testing as the first line of investigation.

2. Types of cell culture

Cell culture broadly denotes maintaining cell population, enabling both growth and propagation of cells. There are different types of cell culture methods. The primary culture involves desegregation of cells from the mother tissue by application of enzymatic or shear processes. Cells are transferred to a sterile system with favorable media for growth. This is usually done in glass or plastic containments such as flasks, petri plates, dishes and so on. Primary cultures are often heterogeneous, that is they are a mixed collection of different cell types that are present in the source tissue. Depending on the application requirements, the mixed pool can directly be considered for a study or the different cell populations may be identified by examining biomarkers and further sorted to obtain a culture of a single type of cells. Primary cultures are often further classified into adherent and suspension cultures. Adherent cultures are anchorage dependent [4]. They require surface support for normal proliferation. Adherent cultures are grown in containers coated with a basal polymeric protein matrix such as lysine.

When normal cells are isolated for a period, detached from the surrounding extracellular matrix, it leads to growth arrest and even the induction of anoikis. The cell-cell contact [5]

http://dx.doi.org/10.5772/intechopen.80818

in the extracellular matrix provides a local niche that provides the necessary growth factors, cytokines and integrin binding molecules that favors cell survival and growth. On the other hand, cells growing as a monolayer in a culture dish, also experience a growth arrest when they have crowded and have no more space to spread out. Cells stop proliferating once they fill out the culture dish. As such routine sub culturing or passaging is necessary, to not only maintain the proliferating pool of cells but also to keep them healthy. Tissue from most organs are candidates for adherent cultures.

Other than primary, there is also the suspension type cultures. These cells do not require a support matrix to grow. Examples include cells of the hematopoietic system. From an industrial perspective, suspension cultures are easier to maintain and implement in a large scale set up. Optimization and quantification of various culture parameters are quicker, and this helps efficient development of protocol for production. Like passaging in adherent cultures, suspension cultures need dilution for further growth and propagation. Cell population in a culture is finite and a function of the concentration of cells in the medium. Constant agitation is required to avoid flocculation of cells. Although do not require enzymatic and mechanical detachment as in adherent cultures. Adherent cultures best suit cytological studies while suspension cultures enhance bulk protein production.

Although primary cells retain genetic integrity of the source tissue, there is a limit to its life span and proliferative ability. It varies between donor tissues, requires optimization of culture conditions and is time consuming to grow. The other alternative to primary are continuous cell lines. They are primary cells transformed through subsequent culturing. Transformation can occur naturally or be induced through chemical and viral means. They greatly benefit from their ease of culturing methods. They are characterized for markers and often are available with well-established protocols of handling and propagation. They enable quicker enable quicker biochemical and cellular analysis of mammalian cells. A large number of experiments can be conducted and repeated to add accuracy to experimental evaluation. This is highly desirable for research and industrial applications. Antibody production, screening of toxic compounds and gene expression studies can all be achieved in a time and cost-efficient manner. Drawbacks however include disparity of investigations with *in vivo* systems. Thus, any use of continuous cell line as a model of study, needs to be followed by *in vivo* analysis and medical trials before consideration for implementation.

3. Cell culture practices

All cultures face challenges of contamination through contact and lack of appropriate sterility techniques. Bacterial and fungal contaminations cloud the culture and shift the pH. pH imbalances also occur due to presence of incorrect salts, bicarbonate buffering and gaseous tension. pH changes may at times result in media precipitation, although this may also be the case for contamination with detergent phosphate used for cleaning the culture vessels and equipment. Contamination with magnesium and calcium ions can lead to cell clumping and lysis particularly in a suspension culture. Increased duration of enzyme treatment such as trypsin not only results in subsequent cell adherence issues but may adversely affect cell's

survivability due impairment of membrane integrity. Cell death can ultimately be induced by several parameters such as fluctuation in the conditioning temperature, CO_2, repeated freeze and thawing of cells, bad cryopreservation techniques, production of toxic metabolites in the culture media etc. [6]. Thus, proper handling and care are vital to cell culture.

Some common cell culture techniques are exercised to ensure maintaining an aseptic environment for culture [7]. These include routine cleaning of designated rooms and facilities for removal of dust and grease. Use of chlorine and xylenol-based disinfectants for open surfaces is another precaution commonly employed. Decontamination of culture premises by fumigation using potassium permanganate and formaldehyde is carried out as per requirement such as the instances of biological contamination. Steam sterilization of all glass and plastic ware, particularly those used for actual culturing within the biosafety cabinet is undertaken. 70% ethanol or isopropyl alcohol is commonly used to swab hands and wipe surfaces during handling. Use of single open glass flame and sterile tissue rolls also supplements this purpose. Double autoclaved and ion free water is to be used to prepare all sterile solutions. Filtration with at least a 0.22-micron pore sized filter is advised to remove biological contaminants in heat labile substances such as trypsin and antibiotic/antimycotic agents. Use of powder free and sterile oil-resistant gloves is recommended where necessary. The laminar air flow system should house a high efficiency particulate air flow system (HEPA filter). A good HEPA filter must be able to retain and remove at least 99% of particles of 0.3 micron in diameter, suspended in the penetrating air. The maximum speed of the filter should not exceed 0.025 m/s as low speed penetration achieves maximum filtration capacity. As a user dependent precaution, it is recommended, to avoid talking during handling, to prevent generation of contaminant carrying aerosols. Proper planning of experiments routes in better execution. Experiments need to be performed as quickly as possible avoiding all unnecessary steps especially those that involve physical contact with the culture.

Once the experiments are conducted, the right and efficient disposal of culture waste is also equally crucial for ethical reasons. Use of 70% alcohol, isopropyl alcohol, sodium hypochlorite (bleach) and autoclaving can all be employed depending upon the material.

Another crucial step to cell culture is the use of ideal media and storage conditions for preservation of cells. Cryopreservation either by storing vials containing cells in −80°C or liquid nitrogen is commonly followed. Components of freezing media are usually serum and dimethyl sulfoxide. Cryovials can be snap frozen by adding them directly to storage conditions or by gradual incubations with decreasing temperatures. The latter is particularly preferred for sensitive cells.

Some safety measures to be considered while dealing with unauthenticated source is to complete quarantine procedures. Particularly new samples need to be tested for mycoplasma, bacterial and fungal contamination. Unless absolutely required, use of antibiotics is not recommended as they may lead to development of resistant strains and may put stress the cultured cells. Sub culturing needs to be done around 80% confluency, to avoid effects of growth arrest by contact inhibition. Cells need to be routinely frozen and revived. Otherwise a continuous culture that runs for months especially for transformed cells has risks of picking up uncharacterized mutations. Care needs to be taken that all reagents used for cell culture

are within the recommended shelf life. All equipment need to be well calibrated and safety cabinets need to be tested for efficacy. Water baths need to be routinely cleaned to avoid contamination. Always sterile water needs to be used in water baths. All work surfaces need to be free of clutter. There must be minimal cardboard packaging if at all required. A splash proof apron and eye protection are to be utilized where necessary.

4. Cell culture-based assays to evaluate toxicity associated with nanoparticles

Nanoparticle formulations to be administered in *in vitro* experiments need to be prepared with care. To avoid inhaling aerosolized nanoparticle, an appropriate pollution mask needs to be worn while handling nanoparticles. It is best to sonicate or vortex and add nanoparticles to allow for dispersion in the nanoscale. This process is called charging. If charging is not done properly, nanoparticle may aggregate and present themselves as micro range particles to the experimental set up. Another precaution to avoid aggregation is to use stock solutions with least possible nanoparticle concentration. Incubation with nanoparticles need to be followed with appropriate washing steps to remove as much particles as possible that have adhered to the cell surface. This prevents interference to the downstream processing of the cell.

One of the first investigations in understanding the effects of any exposing agent is to conduct a cell proliferation assay. Cellular viability, a synonym term is the number of healthy cells in a sample. Viability monitored as a function of dose and time provides information on cell death and hence is also a measure of cytotoxicity [8]. Different parameters indicate the viability of the cell and thus can be used to quantify the cytotoxicity [9]. Some of the methods used to study nanoparticle exposure on cells are described as follows. The dye exclusion methods are a preliminary test based on permeation of dye in dead or dying cells owing to loss of membrane integrity. Trypan blue, eosin and propidium iodide can all be implemented in the dye exclusion test [10]. Dyes are added to cell suspensions and appropriate volumes are loaded onto a counter to aid determination of live cells. Neubauer hemocytometer, a manual counting slide, has been conventionally used to count cells.

Another cell viability assessment is through the documenting the metabolic or enzymatic activity of viable cells. These cells convert the substrate to a colored or fluorescent product and as cell death increases, the degree of this conversion also lacks behind. Examples of this type of tests include protease activity assay and reduction of tetrazolium and resazurin salts [11]. The protease viability assay includes the use of glycylphenylalanyl-aminofluorocoumarin [12]. Abbreviated as GF-AFC, it's a recently developed marker. It permeated live cells and is acted upon by cytoplasmic aminopeptidase. This results in cleavage of glycine and phenylalanine amino acid, releasing AFC (aminofluorocoumarin). AFC generates fluorescent signals corresponding to the number of live cells.

MTT [13] (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) a positively charged tetrazolium dye readily penetrates the cell. It is converted to the colored formazan product by specific NADH dependent mitochondrial enzymes. Formazan crystals accumulate

as insoluble precipitates inside the cell. Cells are lysed, and these crystals are solubilized through combinations of various reagents such as detergent, DMSO, SDS, acidified isopropanol, dimethylformamide, etc. Absorbance readings are then documented, where maximum absorbance is proportional to higher survival. There are some negatively charges tetrazolium dyes such as MTS, XTT, and WST-1. These do not readily penetrate the cell. However, they can still be used for the assay by incorporating an intermediate electron acceptor.

Resazurin (7-hydroxy-10-oxidophenoxazin-10-ium3-one) is particularly used to assay for mitochondrial dysfunction. Resazurin, a deep blue colored complex is reduced to resorufin, a pink colored complex by the enzymes in the inner mitochondrial membrane. Use of resazurin is inexpensive and more sensitive than using tetrazolium dyes.

ATP cell assay is yet another type of evaluation for cell viability. It overrides any incubation with live cell population. It is quicker, more sensitive and less prone to artifacts. Cells with damaged membranes cannot synthesize ATP and endogenous ATPases rapidly depletes cellular ATP concentration. Cells are lysed through detergent activity for this assay, in the presence of ATPase inhibitors to stabilize the total ATP content. Firefly or shrimp derived luciferase acts on substrate luciferin to generate light in the presence of ATP. Higher intensity of light is proportional to the number of live cells.

Sulforhodamine B (SRB) is a fluorescent dye [14]. It is bright pink in color. This aminoxanthene dye binds to cellular protein under mildly acidic conditions. Extraction in a basic environment is proportional to cell mass and thus an indicator of cell viability. Clonogenic cell survival assay investigates the cell's capability for propagation [15]. Treated sample of cells are plated and colonies stained and counted. DNA synthesis cell proliferation assay involves incubating cells with 3H-thymidine. Proliferating cells incorporates this radioactive tracker. Proliferation and thus cell viability can be measured by a scintillation counter. A non-radioactive alternative such as 5-bromo-2′-deoxyuridine (BrdU) can also be implemented. Although this includes an additional step of binding with BrdU-specific antibody and probably a secondary antibody before the colorimetric estimation [16]. A developed 5-ethynyl-2′-deoxyuridine (EdU) can be detected by a fluorescent azide through a Cu(I)-catalyzed cycloaddition reaction. This fast and sensitive method has additional advantages over BrdU assay [17], which are lack of sample fixation and preservation of DNA structure [18]. Raman micro spectroscopy detects variations in Raman bands associated with O–H stretching in water [19]. These variations can non-destructively correspond with ionic concentrations in the intracellular and extracellular fluids. This can be useful in determining the rate and direction of ionic transport. Loss of membrane integrity is dead and dying cells are often associated with leaching of ions, which can be detected and quantified by this technique.

5. Structural tracking as a function of nanoparticle treatment

Morphology modulations can be studied through documenting the changes in the cell as a dose and time dependent function of the exposing agent, using an inverted microscope [20]. Merged with Hoechst stained pictures, can provide additional information on the nuclear

morphology. This blue fluorescent dye is membrane permeable and binds to DNA. Hoechst staining has also been successfully used to track changes in the nuclear integrity, thus documenting the progress of cell fate. Necrosis, apoptosis and cell enlargement have been successfully shown as consequences of different nanoparticle exposure in various cell-based studies.

Phalloidin, a mushroom toxin, with a high affinity for F-actin can be used to track changes in actin dynamics [21]. Fluorescent probes are bound to phalloidin and thus help visualize the actin networks. Gap junctions in neuronal cells can be imaged by using biotinylated dextrans. Biotinylated dextran amines can be introduced in neural cell cultures by pressure injections [22]. Further they can be visualized by avidin conjugated horse radish peroxidase with a metal enhanced diaminobenzidine reaction.

Biocytin hydrazide, an aldehyde-based fixative, is another transneuronal tracer. It detects glycoconjugates and can be used to trace neuronal projections and visualize gap junctions. Biocytin hydrazide [23] in turn is detected by a fluorescent dye conjugated to streptavidin. Cholera toxin subunit B is a protein commonly used to retrograde tracer [24]. It binds to glycosphingolipids in axonal membranes [25]. It can also be used to visualize retinofugal projections. Subunit B is non-toxic and aids internalization and transport of conjugates. Wheat germ agglutinin (WGA) is a lectin protein that binds to N-acetyl-D-glucosamine and sialic acid [26]. Neurons can endocytose WGA-HRP, thus they can be used as tracers as they cross through synapses. Isolectins from legumes *Griffonia simplicifolia* can be used to differentiate between neuronal subtypes [27]. The A subunit prefers N-acetyl-D-galactosamine end groups while the B subunit is selective for terminal a-D-galactosyl residues. Its therefore used as a vascular stain for study of adult neurogenesis. Carbocyanine is a lipophilic dye used to stain plasma membranes [28]. They are highly fluorescent in lipid bilayers while being weakly fluorescent in aqueous phase providing a strong contrast for visualization.

6. Evaluation of key signaling pathways in studying cell fate

Simple but conclusive evaluation of changes in expression of molecules is to conduct a reverse transcriptase polymerase chain reaction (RT-PCR) and carry out a western blot analysis. RT-PCR documents the changes in mRNA (transcriptional) levels, while western blot records changes at the protein (translational) level. Cells exposed to nanoparticles are harvested and thoroughly washed before downstream processing. RNA extracted is converted to cDNA and used as a template for PCR. Appropriate primers are designed for target amplification. Amplicons from the PCR are resolved using agarose gel electrophoresis and visualize by a UV illuminator using ethidium bromide staining.

For western blot analysis, protein is carefully extracted. Whole cell lysate (at least 40 μg) is resolved by SDS-PAGE [29]. Western blot is made by electrophoretic transfer on to membranes. Following transfer membranes are blocked with either non-fat dried milk or BSA. Overnight incubation with primary antibody is usually allowed. Post this, appropriate incubation is carried out with secondary antibody. Number of washes after each step, antibody dilution and incubation time all need to be optimized. Horse radish peroxidase conjugates are commonly

used for color detection or chemiluminescent detection. However, chemiluminescent detection is much more sensitive than color detection. Expression of a target is normalized to an internal control such as β- Actin, to override experimental errors.

Different signaling cascade checkpoints and markers are commonly evaluated to understand the effect of the exposing agent on the cell through RT PCR and western blot analysis [30]. Some of these are highlighted. Akt, protein kinase B is involved in cell proliferation, transcription, migration and glucose metabolism. Caspases particularly, caspase 3 and caspase 9 indicate the progression of apoptosis. LC3B indicates the onset of autophagic processes. Hsp70 and Hsp90 indicate the heightened cellular responses of protein folding to external stresses. Expression of NFκB is an inflammatory response towards survival and cellular propagation. mTOR is directly or indirectly involved in the regulation of protein function.

7. Statistical analysis of experimental data

As important it is to design and execute meaningful investigations in understanding the effects of toxic agents on cellular functions, it is also important to do them with statistical validation. Experiment results need to be accurate and consistent within the framework of the defined system. Reproducibility of protocols and outcomes are important in formulating further scope for any research. In this endeavor, several statistical tools can be employed which include the determination of average, standard deviation, p value and range of error.

Especially with studying toxicity from nanoparticle exposure, the number of exposing agents is exponentially growing every year. As such it becomes time and cost consuming to exact the required number of experiments to test each particle for various routes of exposure. This necessitates the need for development of adequate and accurate prediction tools for toxicity. A raising technique today is the advent of quantitative structure activity-based relationship (QSAR) models [31]. A response curve is defined, and various physiochemical and biological parameters are gathered or determined and fitness to response is evaluated. For *in vitro* experiments the R^2 (correlation coefficient) of any such effort must be greater than 0.81. Such tools help screen large numbers of particles for their toxic outcomes. This ensure only the most promising leads are further evaluated with actual we laboratory experiments, in process, saving resources, time and funds.

8. Conclusion

Accurate risk assessment is an interdisciplinary approach, where acquiring information and processing is a never-ending ordeal. Therefore, an ethical yet smart approach is needed for studying risks associated with nanoparticles. Only the understanding of the mechanisms of toxicity can enable future endeavors towards strategies of safe nanomaterial design and stress recovery solutions.

http://dx.doi.org/10.5772/intechopen.80818

In vivo investigations are largely failing to correlate with clinical trials for various reasons. Many pharmaceutical companies have been using rat, monkey and dog models to evaluate toxicity. These are time consuming and cost intensive with very little productivity in terms of actual drugs reaching the market. *In vitro* investigations have the advantage of being cost and time effective and reduces unethical animal sacrifices and cruelty. Any meaningful result can further be screened out for animal testing, if necessary.

Another advantage of *in vitro* models is that it can be developed into 3D cultures and organ on chip innovations to more accurately predict clinical outcomes [32]. Some recent publications show that the level of prediction is even higher than that of *in vivo* investigations, since there is the scope to study and test human tissue functions along with the mechanical fluidic motions that accurately mimic conditions in the human body.

Thus, it is highly intuitive to encourage young researchers to look at a problem holistically and design best possible routes towards solutions that may lead to implementation and relief. An appreciation of resources and time is vital to avoid wasting them in illogical endeavors. An open mind is to be inculcated that although is enriched with knowledge but chooses to not be limited by it.

Author details

Ansie Martin* and Angshuman Sarkar

*Address all correspondence to: ansiemartin@gmail.com

CMBL, Department of Biological Sciences, Birla Institute of Technology and Sciences, K K Birla Goa Campus, Sancoale, South Goa, India

References

[1] Riss TL, Moravec RA. Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. Assay and Drug Development Technologies. 2004;**2**(1):51-62

[2] Martin A, Sarkar A. Overview on biological implications of metal oxide nanoparticle exposure to human alveolar A549 cell line. Nanotoxicology. 2017;**11**(6):713-724

[3] Parasuraman S. Toxicological screening. Journal of Pharmacology & Pharmacotherapeutics. 2011;**2**(2):74

[4] Merten O-W. Advances in cell culture: Anchorage dependence. Philosophical Transactions of the Royal Society B. 2015;**370**(1661):20140040

[5] Abercrombie M. Contact inhibition and malignancy. Nature. 1979;**281**(5729):259

[6] Ramos TV et al. Standardized cryopreservation of human primary cells. Current Protocols in Cell Biology. 2014;**64**(1):A-3I

[7] Coté RJ. Aseptic technique for cell culture. Current Protocols in Cell Biology. 1998;**1**:1-3

[8] Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. Journal of Immunological Methods. 1989;**119**(2):203-210

[9] Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods. 1983;**65**(1-2):55-63

[10] Strober W. Trypan blue exclusion test of cell viability. Current Protocols in Immunology. 1997;**21**(1):A-3B

[11] Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival: Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. Journal of Immunological Methods. 1986;**89**(2):271-277

[12] Niles AL et al. A homogeneous assay to measure live and dead cells in the same sample by detecting different protease markers. Analytical Biochemistry. 2007;**366**(2):197-206

[13] Bahuguna A et al. MTT assay to evaluate the cytotoxic potential of a drug. Bangladesh Journal of Pharmacology. 2017;**12**(2):115-118

[14] Orellana EA, Kasinski AL. Sulforhodamine B (SRB) assay in cell culture to investigate cell proliferation. Bio-Protocol. 2016;**6**(21)

[15] Munshi A, Hobbs M, Meyn RE. Clonogenic cell survival assay. In: Chemosensitivity. USA: Springer Humana Press; 2005. pp. 21-28

[16] Tada H et al. An improved colorimetric assay for interleukin 2. Journal of Immunological Methods. 1986;**93**(2):157-165

[17] Viggiani CJ, Knott SRV, Aparicio OM. Genome-wide analysis of DNA synthesis by BrdU immunoprecipitation on tiling microarrays (BrdU-IP-chip) in *Saccharomyces cerevisiae*. Cold Spring Harbor Protocols. 2010;**2010**(2) (pdb-prot 5385). http://cshprotocols.cshlp.org/content/2010/2/pdb.prot5385.long

[18] Bhaduri S, Ranjan N, Arya DP. An overview of recent advances in duplex DNA recognition by small molecules. Beilstein Journal of Organic Chemistry. 2018;**14**(1):1051-1086

[19] Puppulin L et al. Raman micro-spectroscopy as a viable tool to monitor and estimate the ionic transport in epithelial cells. Scientific Reports. 2017;**7**(1):3395

[20] Santimano C, Maria, et al. Zinc oxide nanoparticles cause morphological changes in human A549 cell line through alteration in the expression pattern of small GTPases at mRNA level. Journal of Bionanoscience. 2013;**7**(3):300-306

[21] Dancker P et al. Interaction of actin with phalloidin: Polymerization and stabilization of F-actin. Biochimica et Biophysica Acta (BBA)—Protein Structure. 1975;**400**(2):407-414

[22] Reiner A et al. Pathway tracing using biotinylated dextran amines. Journal of Neuroscience Methods. 2000;**103**(1):23-37

[23] Bayer EA, Ben-Hur H, Wilchek M. Biocytin hydrazide—A selective label for sialic acids, galactose, and other sugars in glycoconjugates using avidin-biotin technology. Analytical Biochemistry. 1988;**170**(2):271-281

[24] Trojanowski JQ, Gonatas JO, Gonatas NK. Horseradish peroxidase (HRP) conjugates of cholera toxin and lectins are more sensitive retrogradely transported markers than free HRP. Brain Research. 1982;**231**(1):33-50

[25] Lavail JH, Lavail MM. Retrograde axonal transport in the central nervous system. Science. 1972;**176**(4042):1416-1417

[26] Gonatas NK et al. Superior sensitivity of conjugates of horseradish peroxidase with wheat germ agglutinin for studies of retrograde axonal transport. Journal of Histochemistry & Cytochemistry. 1979;**27**(3):728-734

[27] Ernst C, Christie BR. Isolectin-IB4 as a vascular stain for the study of adult neurogenesis. Journal of Neuroscience Methods. 2006;**150**(1):138-142

[28] Heilemann M et al. Carbocyanine dyes as efficient reversible single-molecule optical switch. Journal of the American Chemical Society. 2005;**127**(11):3801-3806

[29] Sarkar A et al. Antagonistic roles of Rac and Rho in organizing the germ cell microenvironment. Current Biology. 2007;**17**(14):1253-1258

[30] Hynes NE et al. Signalling change: Signal transduction through the decades. Nature Reviews Molecular Cell Biology. 2013;**14**(6):393

[31] Puzyn T et al. Using nano-QSAR to predict the cytotoxicity of metal oxide nanoparticles. Nature Nanotechnology. 2011;**6**(3):175

[32] Huh D et al. A human disease model of drug toxicity-induced pulmonary edema in a lung-on-a-chip microdevice. Science Translational Medicine. 2012;**4**(159):159ra147-159ra147

IntechOpen
IntechOpen

IntechOpen

IntechOpen