Chapter 5. Toxicological studies with cells

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INTRODUCTION

Cell culture provides a model system that provides direct access and evaluation of the effects of chemicals on tissues and constitutes a valuable tool to analyze cell toxicity mechanisms. *In vitro* model systems, in general, have been used to study the mechanism of action of chemical and to analyze the cellular basis for chemicals-induced toxicity as well as to develop rapid and high throughput screening systems for the evaluation of the toxicity of chemicals, which may complement *in vivo* toxicity testing or may replace some *in vivo* models if scientifically validated and accepted by regulatory agencies [1, 2].

The term *in vitro* ("in the glass") refers to the technique of performing a given experiment in a test tube or in a controlled environment outside a living organism. *In vitro* methods are based on the use of cells or tissues which are cultured under controlled conditions in flasks and plates. Cells/tissues are exposed to chemicals and their toxic effect is measured. Increasingly, human cells are used since they better predict possible effects on humans. Commonly used *in vitro* models for assessing chemical toxicity include perfused organ preparations, isolated tissue preparations, single-cell suspensions, and cell-culture systems, such as primary cell cultures and mammalian cell lines. Of these *in vitro* models, cell culture systems have been widely used because they are reliable, reproducible, and relatively inexpensive experimental systems to assess chemical toxicity at the cellular level [3]. Nevertheless it should be pointed out that whether *in vitro* tests are based on primary cells, immortalized (e.g., SV40 transformation) and cancer-derived cell lines, stem cells, or reconstituted tissue cultures, it is important to have *in vitro* systems that adequately mimic key events of the *in vivo* mechanisms of action triggered in humans upon exposure to a toxic compound [4].

Safety testing of chemicals is required under several directives of the European Union (EU) and international regulatory environments. At an international level, the OECD (Organisation for Economic Cooperation and Development) is

developing a Guidance Document on Good In Vitro Method Practices [5] which aims to ensure that efficacy and efficiency of the process between *in vitro* method development and its implementation for regulatory use [6]. The first guidance report on good cell culture practice was released in 2005 [7] and a second one related with the use of stem cells in 2017 [8] and some of the recommendations exposed in these reports are summarized below.

IN VITRO CULTURE CONDITIONS

Cells or tissues in culture require specific conditions that differ from *in vivo* systems. However, cell culture conditions vary for each cell type. The consequences of deviating from the culture conditions required for a particular cell type can range from the expression of aberrant phenotypes to a complete failure of the cell culture [7]. Specific elements of culture conditions include culture media, supplements and other additives, culture-ware and incubation conditions.

- **Basal medium:** refers to a complex nutritive media designed to obtain a good cell viability or cell proliferation or to maintain a desired cell differentiation. Many solid or liquid medium formulations are commercially available with subtle changes in their medium formulation (including phenol red, glutamine and other additives). Most of the commercial media are derived from the Eagle´s minimum essential medium (MEM) which contains amino acids, salts, glucose and vitamins, or its modifications such as Dulbecco's Modified Eagle's medium (DMEM) which contains a four-fold higher concentration of amino acids and vitamins, as well as additional supplementary components and was initially developed for the culture of embryonic mouse cells. The Ham´s nutrient mixtures were originally developed to support the growth of several clones of Chinese hamster ovary (CHO) cells, as well as clones of HeLa and mouse L-cells. The RPMI media (developed by Moore et. al. [9] at Roswell Park Memorial Institute, hence the acronym RPMI), uses a bicarbonate buffering system and alterations in the concentrations of amino acids and vitamins and has been used for the culture of human normal and neoplasic leukocytes. However, their supplementation has demonstrated wide applicability for supporting growth of

many types of cultured cells, including fresh human lymphocytes. However, even subtle changes in the media formulation can alter the characteristics of certain cells or tissues, therefore the medium to be used should be precisely specified.

- **Serum:** is a complex mixture essential for the maintenance and/or proliferation of many cell types. However, due to its complexity and batch-to-batch variations introduces unknown variables in the culture system and in addition it represents a potential source of microbiological contaminants such as mycoplasma and bovine viruses due to its animal origin [7].
- **Serum free media** are commercially available in order to decrease the batch to batch variability problems associated with the use of serum and offer better reproducibility and the potential for selective culture and differentiation of specific cell types such as cell lines [10]. However, serum free supplements can also include poorly defined components such as pituitary extracts, chick embryo extracts, bovine milk fractions or bovine serum albumin (http://www.oecd.org/env/ehs/testing/OECD%20Draft%20GIVIMP_v05%20-%20clean.pdf) which can exhibit batch to batch variation in biological activity.
- **Antibiotics:** these agents are used in cell culture to protect against contamination and for the selection of recombinant clones that express antibiotic resistant genes. The use of antibiotics should be avoided if possible or minimized since those agents can interfere with normal cell biology [7].
- **Cell culture matrix**

  All cell culture plastic or glasses need to be steril and are usually disposable plastic culture vessels commercially available. Most of the culture vessels are manufactured from polystyrene, a long carbon chain polymer with benzene rings attached to every other carbon. Polystyrene was chosen because it had excellent optical clarity and could be sterilized by irradiation. However, it is very hydrophobic and may difficult cell attachment. Therefore, to increase cell attachment to the plastic surface, polystyrene is usually treated to create a more hydrophilic surface and negatively charged after medium addition. However, some cells are not able to attach in polystyrene treated surfaces and require the coating with biological materials including extracellular matrix, attachment and adhesion proteins (collagen, laminin and fibronectin) and mucopolysaccharides, such as heparin sulfate, hyaluronidate and chondroitin sulfate, both individually

and as mixtures. Another useful coating surface is the synthetic polymer poly-D-lysine (PDL) which creates a positive charge on polystyrene and consequently, for some cell types, enhances cell attachment, growth and differentiation, especially in serum-free and low serum conditions [11]. However, most of the coated culture surfaces need to be washed before cell seeding to avoid the possible toxicity of the coating material [7]. Figure 5.1 shows images of commercial presentations of plastic cultures (flask and plates), commercial culture medium and culture plates after coating with laminin and PDL and washed with sterile phosphate buffered saline.

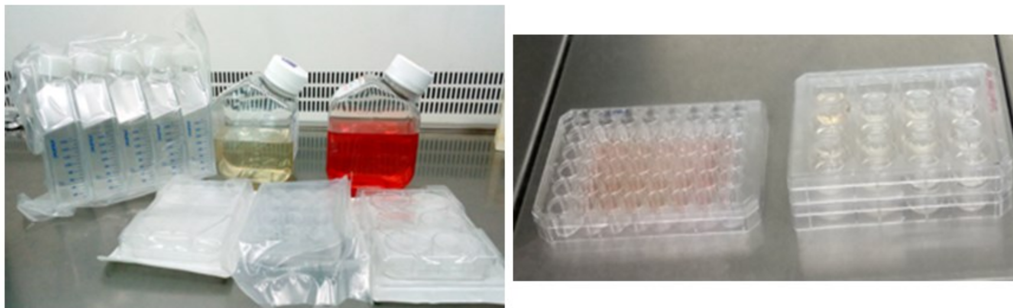


Figure 5.1. Images showing different formats of plastic culture vessels (flasks and plates) and commercial culture media (left panel) and precoated and washed culture plates ready for cell culture (right panel).

HANDLING AND MAINTENANCE OF CELL CULTURES

Cell lines and primary tissues may contain microorganisms or pathogens able to cause human diseases or alter the *in vitro* results. To avoid these hazards, cell culture should be handled at biosafety (hazard) level 2. As a minimum, cell culture should be performed in a class II biological safety cabinet (Figure 5.2). Class II cabinets are designated to prevent biological exposure to personnel and the environment and to protect experimental material from being contaminated. Biological safety cabinets use high efficiency particulate air (HEPA) filters in their exhaust and/or supply systems. These filtered cabinets are primarily designed to protect against exposure to toxics, including biological agents used in the cabinet. Air flow is drawn from the room around the operator into the front grille of the cabinet, which provides personnel protection. In

addition, the downward laminar flow of HEPA-filtered air provides protection for experimental material inside the cabinet. Because cabinet air has passed through the exhaust HEPA filter, it is contaminant-free, providing environmental protection, and may be recirculated back into the laboratory (Class II Type A biological safety cabinets) or ducted out of the building (Class II Type B biological safety cabinets). A scheme of the air flow through a biological safety cabinet is shown in Figure 5.2.

In addition, cell or tissues in culture should be kept in controlled ambient conditions. The optimal culture temperature depends on the cells type, thus insect cells have low optimal growth temperatures than mammalian cells while most mammalian cells growth normally well a temperatures of 37 ºC [7]. Moreover, oxygen and carbon dioxide are vital for cell growth and for most cell types the appropriate atmosphere is normally 5% v/v carbon dioxide in air. Cell culture incubators maintain a constant temperature and high humidity for the growth of tissue culture cells under a CO_2 atmosphere. Typical control of temperature settings in the cell culture incubator range from 4 ºC to 50 ºC, and CO_2 concentrations run from 0.3 to 19.9 %. Moreover, the temperature is typically controlled either by a water bath that circulates through the walls of the cabinet, or by electric coils that give off radiant heat. Some units also include refrigeration for cooling. Relative humidity is maintained between 95% and 98% by an atomizer system or a water reservoir. An image of a cell culture incubator is shown in Figure 5.2.

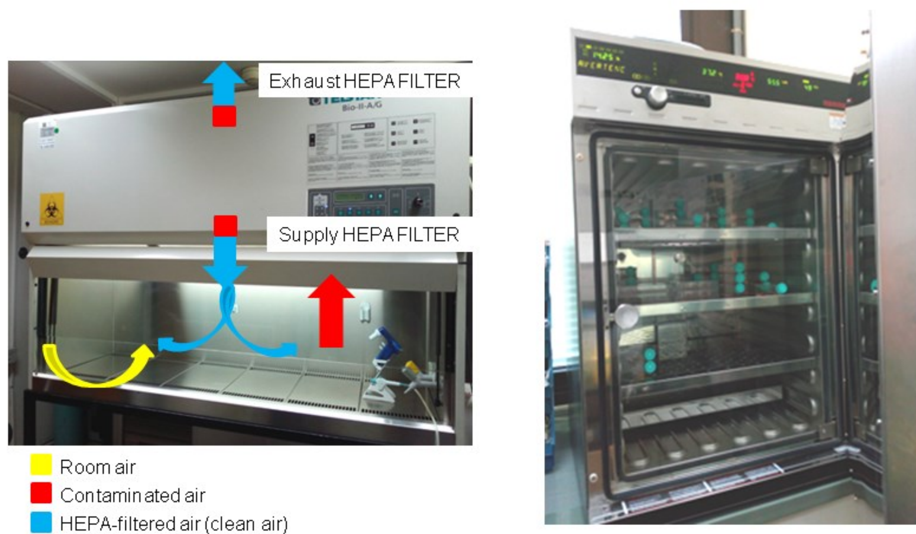


Figure 5.2. Images showing air flow through a biological safety cabinet for handling of cell cultures (left) and a culture incubator (right).

IN VITRO CULTURE MODELS

In vitro model systems are frequently grouped in three broad groups listed below.

1. **Isolated organs or tissues:** these cells are normally obtained directly from an animal or a donor. These i*n vitro* systems are widely used in toxicological applications and may be used in different preparations that include:

a. **Slices of certain tissues** (liver, lung, kidney, brain) that, temporarily, retain some structural and functional features of the original organ.

b. **Isolation and reagregation of cells from different organs** given rise to two or three-dimensional cultures that also retain some functional properties of the original organ and tissue.

c. **Cells from blood or other body fluids** can be prepared as homogeneous preparations and kept *in vitro* for several days, or even used to generate stem cells (umbilical cord cells, bone marrow cells). An image showing the steps for purification of human T lymphocytes in our laboratory [12] as well as a confocal image for the expression of the voltage-dependent potassium channel KCNC1 (or $K_v3.1$) in control human T lymphocytes and in the same cells after 48 hours of treatment with the lecitin concanavalin A at 50 µg/ml is shown in Figure 5.3.

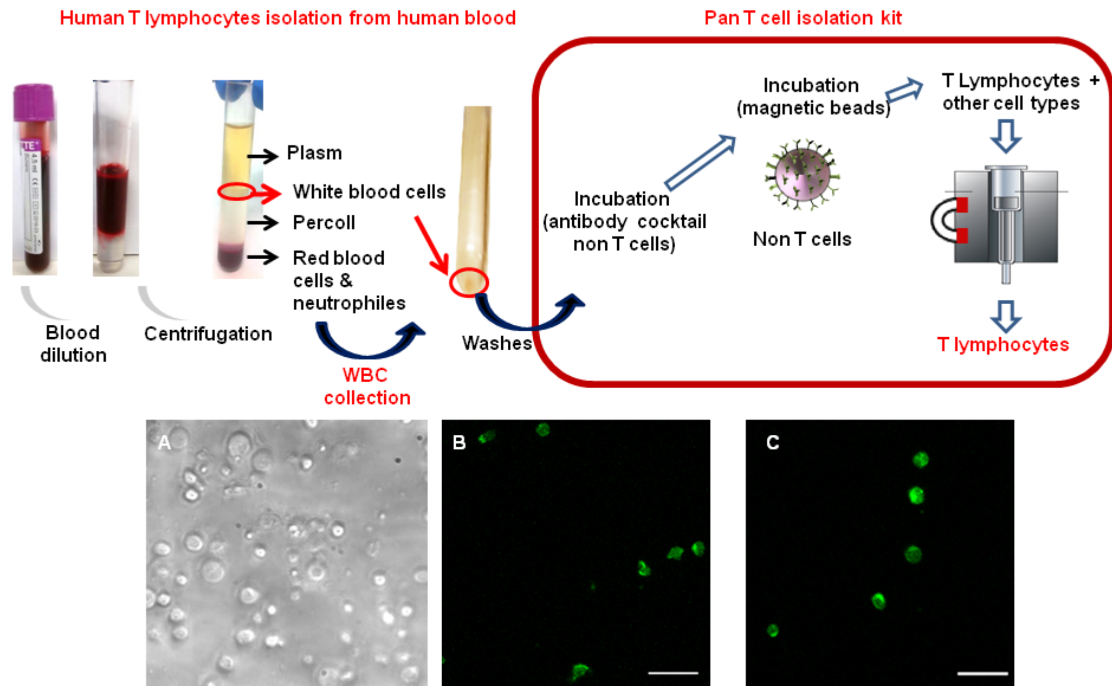


Figure 5.3. Upper panel: scheme showing the isolation of human T lymphocytes from human blood donors. Lower panel: Phase contrast microscope image of purified human T lymphocytes (A). B. Confocal image showing the expression of the Kv3.1 potassium channel in human T lymphocytes cultured for 48 hours, and (C) the expression of the same channel after treatment of the cells for 48 hours with concanavalin A. WBC; white blood cells.

2. **Primary cultures and early passage cultures:** These *in vitro* cultures are harvested cells and tissues obtained directly from animals or humans but maintained *in vitro* for several times depending on the cell type. Frequently, those cultures retain key morphological and functional features of the *in vivo* system and are widely used for basic research and in different *in vitro* applications. Depending on the tissue of origin cells in primary culture can proliferate, however they life-span is limited and may change their initial characteristics with time in culture. With some exceptions, these systems normally represent heterogeneous cell populations that may be maintained either in suspension or in monolayers in glass or plastic surfaces. Examples of such cultures are primary cultures of cortical or cerebellar neurons widely used for the study of toxicity and mechanism of action of neurotoxic compounds [13] such as marine toxins [14-16]. Figure 5.4 shows a scheme for the isolation of primary cultures of cortical neurons and the *in vitro* development of such cells.

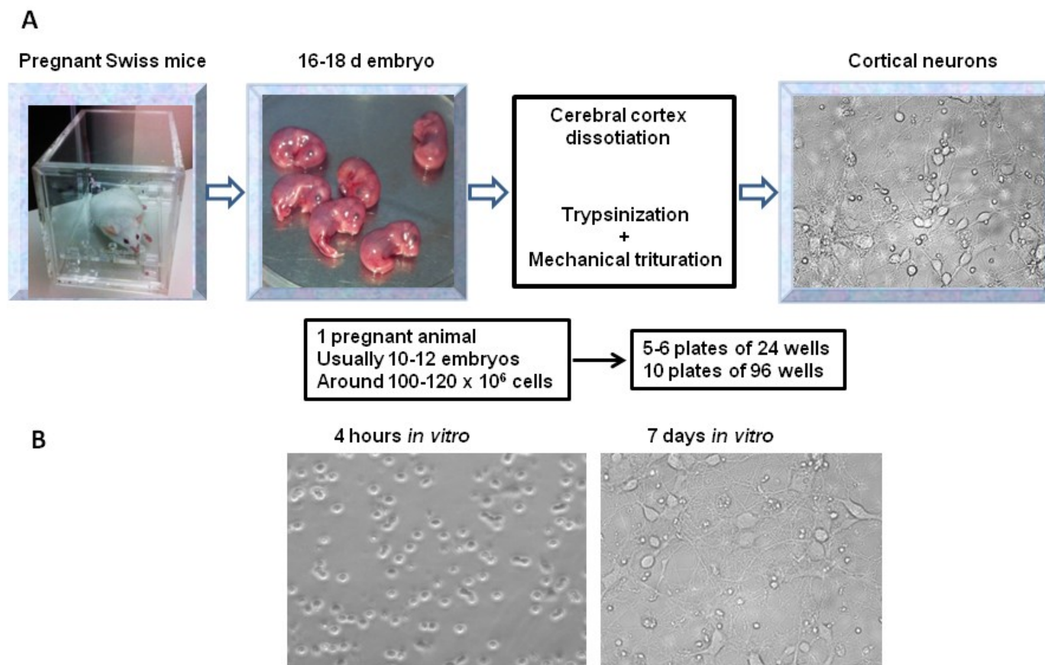


Figure 5.4. Scheme illustrating the procedure for the isolation of primary cultures of cortical neurons (A). Primary cultures of cortical neurons develop their typical morphology and functional characteristics with time in culture (B)

3. **Cell lines:** this term refers to cells that are able to multiply *in vitro* for extended periods of time and can be maintained in subculture. Cell lines can be divided in finite cell lines, continuous cell lines and stem cell lines.

a. **Finite cell lines:** refer to cell lines that can be subcultured or passaged for a period of time but finally the cells stop to replicate but still maintain viability. Numerous finite cell lines have been established and they are genetically stable and remain diploid for many passages but generally reach senescence after 50-60 passages.

b. **Continuous cell lines**: those are cells that can be subcultured indefinitely and do not reach senescence. These are cells usually derived from tumors or normal embryonic tissues. These cell lines can either generate spontaneously or can be produced through a variety of techniques including radiation or treatment with chemical mutagens or carcinogens, isolated from cultures infected with viruses, through genetic modification by transfection or obtained from transgenic animals.

Widely used cell lines include epithelial cell lines obtained from the ovary of an adult Chinese hamster (CHO cells) or the human liver cancer cells HepG2 shown in Figure 5.5.

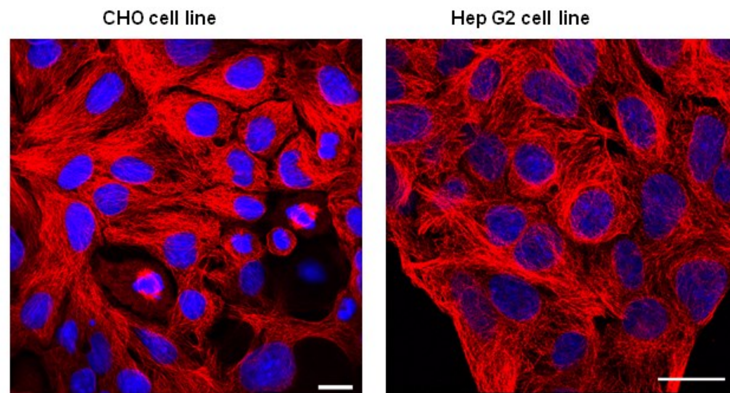


Figure 5.5. Confocal microscopy images showing adherent CHO cells and HepG2 cells after immunocytochemistry for the cytoskeleton marker tubulin (red). Nuclei were labeled with the nucleic acid stain Hoechst (blue). Images were kindly provided by Dr. J.A. Rubiolo (University of Santiago de Compostela)

c. **Stem cell lines**: are continuous cell lines that retain the characteristics of stem cells and can produce diverse differentiated cell types. They require great care in their maintenance, handling and preservation. One example of a human stem cell line is the CTX0E16 cell line which is a human neuronal stem cells line, obtained from the cerebral cortex of a fetus of 12 weeks of gestation and immortalized by the ectopic expression of the c-mycER^{TAM} transgene and kindly provided to our laboratory by a material transfer agreement with ReNeuron Limited (Guildford, Surrey GU2 7AF, U.K). Human neural progenitor cells CTX0E16 hNPCs were cultured following the provider instructions as previously reported [17]. Briefly, proliferating cells were maintained in reduced modified medium (RMM) containing DMEM: F12 with 15 mM HEPES and sodium bicarbonate (Sigma) supplemented with 0.03% human serum albumin (Sigma), 100 µg/ml apotransferrin (Scipac Ltd , Kent, UK), 16.2 µg/ml putrescine (Sigma), 5 µg/ml human insulin (Sigma), 60 ng/ml progesterone (Sigma), 2 mM L-glutamine (Sigma) and 40 ng/ml of sodium selenite (Sigma). Under proliferative conditions cells were cultured in the presence of 10 ng/ml of human fibroblast growth factor (FGF2), 20 ng/ml of human epidermal growth

factor (EGF), both from PeproTech, Rocky Hill, NJ and 100 nM hydroxy tamoxifen (4-OHT, Sigma). CTX0E16 hNPCs were seeded onto Poly-D-lysine (PDL, 5 µg/cm^2, Sigma,) and laminin-coated (1 µg/cm^2; Sigma) tissue culture flasks, with full media changes occurring every 2–3 days. Cells were passaged once they reached 70–80 % confluence using Accutase (Sigma) and maintained between 25 and 30 passages; all experiments were carried out using cells from passages 12 to 30. For differentiation CTX0E16 cultures were washed twice with non-supplemented DMEM:F12 medium and passaged onto PDL and laminin-coated tissue culture plates or glass coverslips at a density of 50,000 cells per ml. Cells were then washed in warm Dulbecco's phosphate-buffered saline (DPBS: Thermofisher) and maintained in neuronal differentiation media (NDM: Neurobasal Medium (Thermofisher) supplemented with 0.03 % human serum albumin, 100 µg/ml apotransferrin (Scipac Ltd, UK), 16.2 µg/ml putrescine (Sigma,), 5 µg/ml human insulin (Sigma,), 60 ng/ml progesterone (Sigma,), 2 mM L-glutamine (Sigma), 40 ng/ml sodium selenite (Sigma,) and 1 × B27 serum-free supplement (Thermofisher). Half medium changes were performed every 2–3 days and cultures were differentiated for up to 60 days (days differentiated (DD)). Undifferentiated and differentiated phase contrast microscopy images of these human neuronal stem cells are shown in Figure 5.6.

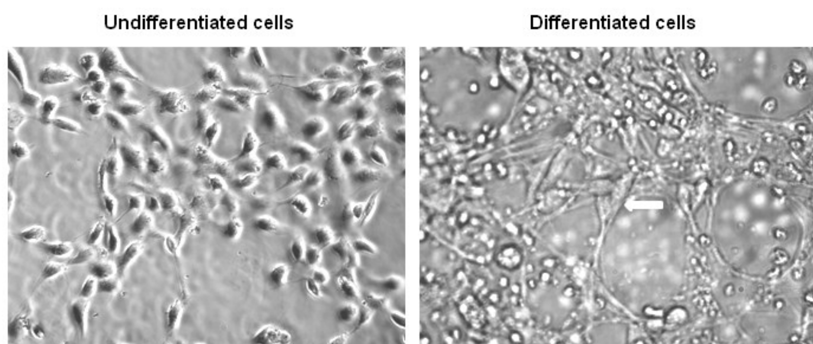


Figure 5.6. Phase contrast image showing undifferentiated (left) and differentiated human neuronal stem cells (right). Arrow heads indicate neuronal cell bodies.

IN VITRO CYTOTOXICITY TESTS AND METHODS TO EVALUATE CELLULAR FUNCTION

In vitro tests may measure cellular function and cell death. Cell function is normally determined by the evaluation of the effect of chemicals on events or cellular signaling cascades that are related to cell injury or toxicity although several cell death tests are commonly used to evaluate cellular toxicity [3]. *In vitro* methods are common and widely used for screening and ranking chemicals, and have also been taken into account sporadically for risk assessment purposes in the case of food additives, however, a major promise of *in vitro* systems is to obtain information on the mechanism-of action of chemical which is considered pivotal for adequate risk assessment [18].

In Europe the European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM) has been established in 2011 and promotes the development and validation of methods alternatives to animal testing. This laboratory has promoted the creation of the EURL ECVAM DataBase Service on ALternative Methods to animal experimentation (DB-ALM), a public and freely available service that provides evaluated information on the development and applications of advanced and alternative methods to animal experimentation in biomedical sciences and toxicology, both in research and for regulatory purposes (https://ecvam-dbalm.jrc.ec.europa.eu/). Actually, this database has evaluated more than 300 methods and almost 200 protocols, following the OECD guidelines. In total, detailed DB-ALM method descriptions, presented as comprehensive method summaries or individual protocols, are available for 26 different topic areas. Since, the recompilation of all the available *in vitro* methods is out of the scope of this chapter, some of the methods are listed accordingly to the proposed endpoint following the DB-ALM classification and other methods are listed in more detail, focusing on those frequently used in our laboratory to analyze to toxicity of marine toxins.

Usually, cytotoxicity is considered primarily as the potential of a compound to induce cell death and is mostly frequently related to necrosis, a term used to define a class of irreversible cell death that most often results from acute cellular injury that causes metabolic failure of the cell that coincides with rapid depletion of ATP [19]. Most *in vitro* cytotoxicity tests measure necrosis. However, an equally important mechanism of cell death is apoptosis, also

known as “programmed cell death” which is a type of cell death that is mediated by a genetically controlled, energy-requiring program which requires different methods for its evaluation [19]. The inhibition of apoptosis is also of toxicological importance. Furthermore, detailed studies on dose and time dependence of toxic effects to cells, together with the observation of effects on the cell cycle and their reversibility, can provide valuable information about mechanisms and type of toxicity, including necrosis, apoptosis or other events [18]. It is widely accepted that *In vitro* cytotoxicity tests are useful and necessary to define basal cytotoxicity, for example the intrinsic ability of a compound to cause cell death as a consequence of damage to basic cellular functions. Cytotoxicity tests are also necessary to define the concentration range for further and more detailed *in vitro* testing to provide meaningful information on parameters such as genotoxicity, induction of mutations or programmed cell death. By establishing the dose at which 50 % of the cells are affected (i.e. $TC_{50;}$ TC: toxic concentration), it is possible to compare quantitatively responses of single compounds in different systems or of several compounds in individual systems [18].

IN VITRO METHODS TO EVALUATE THE EFFECTS OF COMPOUNDS ON REPRODUCTION

As mentioned above, the *in vitro* methods included in the DB-ALM database are listed in function of the endpoint analyzed, and each endpoint may include or not several subgroups. For example, the *in vitro* methods to evaluate the effects of compounds on reproduction are divided in three categories analyzing male and female fertility and developmental toxicity (Figure 5.7), however, as stated in the review document of these methods none of these *ex vivo/in vitro/ in silico* methods have yet gained regulatory acceptance to date [20]. For this reason, these alternative methods do not allow for full replacement of animal testing. Therefore, these methods are mostly used for screening purposes and are intended to be applied in test batteries and as part of integrated testing strategies. However, with reference to "Reduction and Refinement" of animal use, the Extended One-Generation Reproductive Toxicity Study (EOGRTS) has been adopted as OECD Test Guideline 443 [21]. For male and female fertility

the list of cell based protocols is summarized in Figure 5.7 while for developmental toxicity only the protocols are listed.

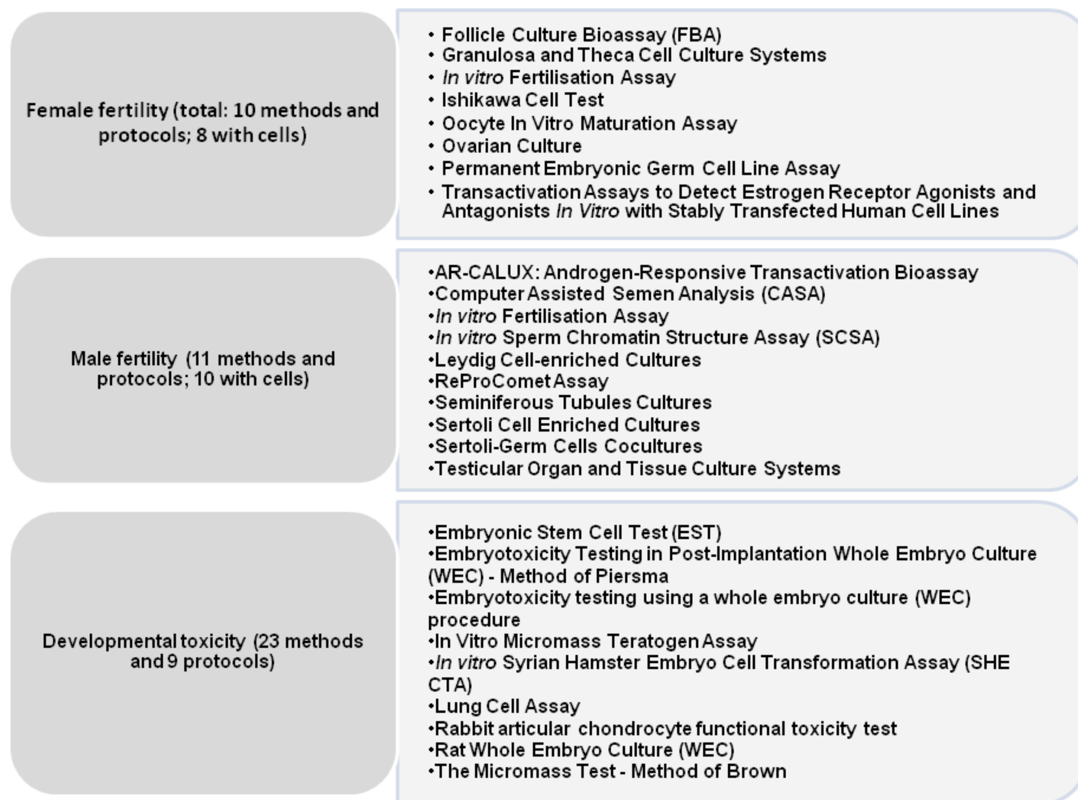


Figure 5.7. List of the reviewed *in vitro* methods to assess the effects of compounds on reproduction.

IN VITRO METHODS IN CANCER RESEARCH

Cancer research and carcinogenicity and tumor promotion is one of the priority topics established in the DB-ALM database. For the field of cancer research, the methods are divided in three categories that include drug discovery and activity testing (6 methods), carcinogenicity (4 methods) and tumor promotion (1 method). The methods included in this section are listed in Figure 5.8.

Drug discovery and activity testing (6 methods)

- Annexin V assay
- Colorimetric Cytotoxicity Assays for Anchorage Dependent Cells (MTT based
- DNA fragmentation stains using the bisbenzimide dyes (Hoescht 33258 and Hoescht 3334)
- Determination of DNA fragmentation with 4'-6-diamidine-2-phenylindole (DAPI) staining
- Diphenylamine assay
- Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) assay

Carciogenicity (4 methods)

- Alkaline Unwinding Genotoxicity Test
- Bhas 42 Cell Transformation Assay in 6- and 96-well plates
- Cell transformation assay with BALB/c 3T3 cells (BALB/c 3T3 CTA)
- *In vitro* Syrian Hamster Embryo Cell Transformation Assay (SHE CTA)

Tumour promotion (3 methods)

- Lucifer Yellow Intercellular Exchange Assay for Tumour Promotors
- Screening System of Promoters Using RAS Transfected BALB 3T3 Clone (Bhas 42)
- Serum-Free Liver Mitogen Test

Figure 5.8. List of the reviewed *in vitro* methods for cancer research.

IN VITRO METHODS FOR ENVIRONMENTAL TOXICITY

For testing the environmental toxicity of chemicals and biological agents the DB-ALM provides several test divided in different sections that include Aquatic Short-Term Toxicity (6 methods), Genotoxicity/Mutagenicity (11 methods and 8 protocols), Haematotoxicity (1 methods and 1 protocol), Hepatotoxicity / Metabolism-mediated Toxicity (31 methods and 9 protocols, however under this epigraph several methods for cell culture are included) and 1 method and 1 protocol for immunotoxicity. A summary of the DB-ALM methods to test for environmental toxicity is provided in Figure 5.9.

Category	Methods
Aquatic Short-Term Toxicity (6 methods)	•Allium Test • Fish Embryo Acute Toxicity Test with Zebrafish •Tetrahymena Assay for Membrane-Stabilising Activity •*Tetrahymena* Proliferation Rate and Maximal Density •*Tetrahymena thermophyla* Chemosensory Response •The *Dunaliella tertiolecta* Test, a Marine Algal Assay
Genotoxicity/Mutagenicity (11 methods, 8 protocols)	•Prostaglandin H Synthase (PHS) - Mediated Genotoxicity of Xenobiotics •Bacterial Mutation Assay (with *S.typhimurium* and *E. coli*) •Bacterial reverse mutation test (Ames test) •Cytotoxicity and Genotoxicity in Primary Cultures of Human Hepatocytes •DNA Binding Studies for Alkylating Compounds Using Isolated Perfused Rat Liver •DNA Binding in Bacteria •GreenScreen HC™ Genotoxicity Test •Permanent Embryonic Germ Cell Line Assay •Unscheduled DNA Synthesis in Hepatocyte Cultures Assessed by the Nuclei Procedure
Haematotoxicity (1 method)	•Colony Forming Unit-Granuloyte/Macrophage (CFU-GM) Assay
Hepatotoxicity / Metabolism-mediated Toxicity (31 methods)	•Alginate entrapped primary hepatocytes (LiverBeads™) •CYP1A1-Inducing Potency and Cytotoxicity Test in the Hepa-1 Mouse Hepatoma Cell Line •H-4-II-E Rat Hepatoma Cell Bioassay •Liver Slice Hepatotoxicity Screening System •Rat Hepatocyte Flow Cytometric Cytotoxicity Test •Reactive Metabolite Formation by Fortified Liver Microsomes •The use of avian embryonic tissue for studies on metabolism and metabolism-mediated toxicity of chemicals •The use of stem or progenitor cell-derived hepatocyte-like cells •The use of subcellular fractions of liver tissue and liver homogenate •Use of Stable Cell Lines Expressing Cytochromes CYP cDNA in Toxicity Testing
Immunotoxicity	•Polymorphonuclear Leucocytes Locomotion

Figure 5.9. List of the reviewed *in vitro* methods to assess the environmental toxicity of compounds.

IN VITRO METHODS FOR BASAL TOXICITY AND CYTOTOXICITY

Under this section about 17 methods were reviewed and approved/recommended by the experts of the DB-ALM to test for the basal toxicity and cytotoxicity of compounds and biological agents, including marine toxins. These methods are detailed below summarizing the cell type employed and the cellular function or activity evaluated. Since this group of methods are usually employed as starting points are examined.

Chinese Hamster Ovary (CHO) Cell Proliferation Test and CHO Cell Na^+/K^+/ATPase activity test: These are two different methods using the same cellular model. CHO cells constitute a system useful for ecotoxicological studies. The proliferation rate of CHO cells correlates with physiological membrane functions, such as adenosine uptake and the activity of Na^+/K^+/ATPase. In the proliferation assay CHO cells are cultured for one week in Petri dishes with various concentrations of test substance and the number of cells is counted twice daily, and the proliferation rate is determined from the

logarithmic growth phase. The CHO cell Na^+/K^+/ATPase test measures the activity of the enzyme in the membrane of CHO cells.

Cytoskeletal Alterations as a Parameter for Assessment of Toxicity: the method is based on the determination of changes of cytoskeletal proteins (α and β tubulin and vimentin) after exposure to compounds by indirect immunofluorescence microscopy or quantitative biochemical methods (extraction of tubulin from the cells and measuring the tubulin content of the extracts using a colchicine binding assay). Immunocytochemistry and immunofluorescence microscopy is a useful method to evaluate the effects of marine toxins on cell integrity [22] and a protocol for a such a methods is shown in Figure 5.10.

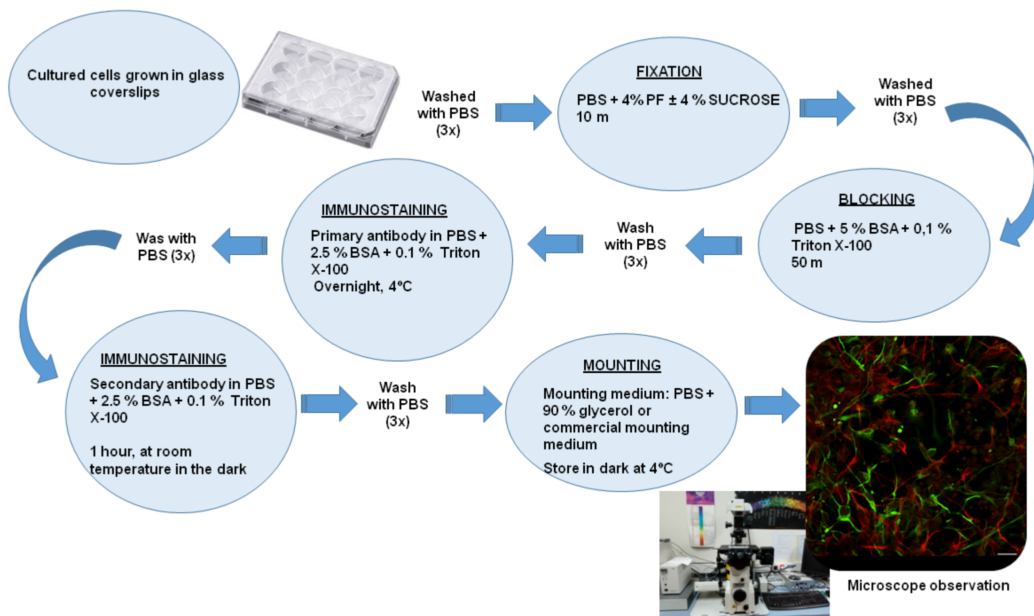


Figure 5.10. Scheme showing the protocol for immunostaining of adherent cells in culture.

The main advantage of this method is that microscope visualization of cytoskeletal proteins provides a detailed view of changes in morphology, however the technique is not adequate to quantify for protein expression changes. An example of the results obtained after the treatment of neuronal cells with chemicals and the analysis of their effect on the cytoskeleton is shown in Figure 5.11. In the upper panel control cortical neurons of 7 days in culture and age-matched neurons were treated with the marine ciguatoxin CTX3C for

24 hours and a double staining was performed with antibodies against the neuronal microtubule associated protein MAP2 and against the GluR2/3 subunit of glutamate receptors. No apparent changes in protein expression or cell morphology were observed. In contrast, in the lower panel, human neuronal stem cells were treated with vehicle or with active derivative of the neurotoxins 6-hydroxydopamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) known as 1-methyl-4-phenylpyridinium ion (MPP^+), which is able to induce degeneration in human neuronal stem cells [23] and after were stained with the neuronal cytoskeletal marker b3-tubulin and the neuronal nuclear marker NeuN. As shown in Figure 5.11 morphological changes were evident in cells treated with the neurotoxin MPP^+.

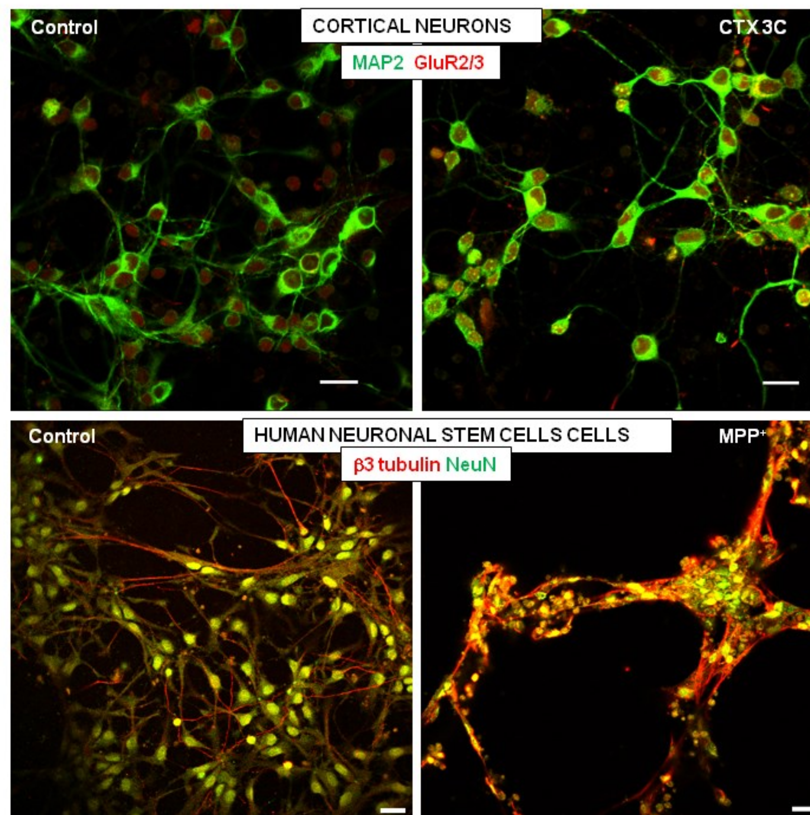


Figure 5.11. Confocal microscope images showing the staining of cytoskeletal proteins in primary cultures of cortical neurons (upper panel) and human neuronal stem cells (lower panel). Cultured cortical neurons non treated (control) and treated with the marine toxin CTX3C were stained with MAP2 (green) and the glutamate receptor subunit GluR2/3 (red), showing that the toxin did not alter neuronal morphology. In the lower panel human neuronal stem cells were exposed to the neurotoxin MPP^+ and stained with the neuronal cytoskeletal marker β3 tubulin

(red) or the nuclear protein NeuN (green). MPP^+ treatment caused a dramatic change in cell morphology.

As mentioned above, one the main disadvantages of immunocytochemistry is its difficulty to objectively quantify changes in protein expression levels. The data shown in the upper panel of Figure 5.11 does not indicates any change in the level of expression of the glutamate receptors subunit GluR2/3 after ciguatoxin treatment in cortical neurons. However quantitative analysis of the expression levels of GluR2/3 by western blotting showed a significant decrease in GluR2/3 expression in cortical neurons after ciguatoxin treatment [15].

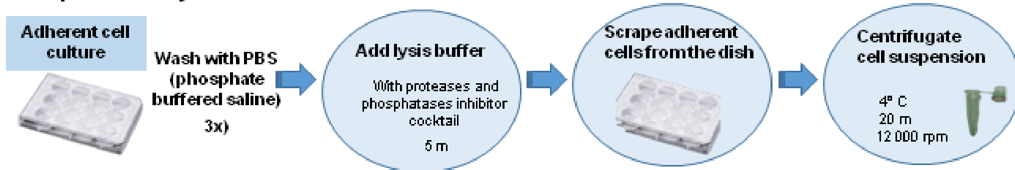
Western blotting is an important technique used in cell and molecular biology to identify specific proteins from a complex mixture of proteins extracted from cells and it allows to quantify the protein expression as well [24].The technique uses three elements to identify a specific protein that include:
-Separation of the protein by size or molecular weight
-Transfer the proteins to a solid support
-Identification of proteins using primary and secondary antibodies.
Although several different methods can be used to quantify proteins in biological samples, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used analytical method to resolve separate components of a protein mixture. The technique is used by many laboratories to investigate or demonstrate expression changes of a given protein between control states and experimental conditions and is still the method of choice for basic research, but also a useful tool in clinical applications and may be applied to any biological sample including cell or tissue extracts and body fluids, such as plasma or serum or urine. [25]. A scheme of the procedure used for western blot in lysates from adherent cell cultures is shown in Figure 5.12. In brief, cell lysates are one of the most common systems used in western blot. Protein extraction from mammalian tissues usually requires mechanical disruption and the use of reagents containing detergents. Moreover, cell lysis should be performed at low temperature and with protease and phophatase inhibitors to prevent protein degradation. Once extracted, protein concentration is measured, usually with a spectrophotometer to quantify the amount of protein loaded into each well. Afterwards, the sample is diluted into a loading buffer,

which contains glycerol that increases the density of the sample relative to the surrounding running buffer making it easier to load in the well, a tracking dye (bromophenol blue) used to follow the run of protein sample on the gel, sodium dodecy sulphate (SDS) to denature proteins and load the proteins with a strong negative charge that will allow each protein to migrate in the electrophoretic field in a measure proportional to its size and thiol reagents (β-mercaptoethanol) to reduce disulfide bonds. It is also very important to have positive and negative controls for the sample. For a positive control a known source of target protein, such as purified protein or a control lysate is used. This helps to confirm the identity of the protein, and the activity of the antibody. A negative control is a null cell line, such as β-actin, is used as well to confirm that the staining is not nonspecific. Western blot uses two different types of agarose gel: stacking and separating gel. The higher, stacking gel is slightly acidic (pH 6.8) and has a lower acrylamide concentration making a porous gel, which separates protein poorly but allows them to form thin, sharply defined bands. The lower gel, called the separating, or resolving gel, is basic (pH 8.8), and has a higher polyacrylamide content, making the gel's pores narrower. Protein is thus separated by their size more so in this gel, as the smaller proteins to travel more easily and hence rapidly, than larger proteins. After separating the protein mixture, it is transferred to a membrane (blotting) using an electric field oriented perpendicular to the surface of the gel, causing proteins to move out of the gel and onto the membrane. In this step it is necessary to ensure a close contact between the gel and the membrane and the placement of the membrane between the gel and the positive electrode. The membrane must be placed as such, so that the negatively charged proteins can migrate from the gel to the membrane. This type of transfer is called electrophoretic transfer and can be done in semi-dry or wet conditions. Wet conditions are usually more reliable as it is less likely to dry out the gel and is preferred for larger proteins while semi-dry transfer is faster. Membranes are made either of nitrocellulose or polyvinylidene difluoride (PVDF). Nitrocellulose is used for its high affinity for protein and its retention abilities but does not allow the membrane to be used for reproving. In contrast, PVDF membranes provide better mechanical support and allow the blot to be reproved and stored but have a higher background than nitrocellulose membranes. Regarding the washing, blocking and antibody

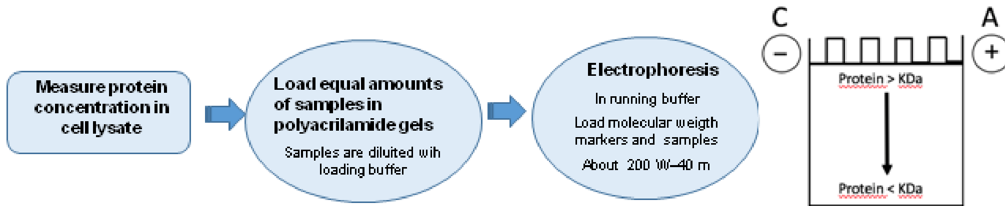
incubation steps, it should be mentioned that blocking is very important to prevent antibodies from binding to the membrane in a non specific manner. Blocking is usually performed in 5% bovine serum albumin (BSA) or nonfat dried milk to reduce background. Frequently the concentration of the antibody used are chosen following the instructions provided by the manufacturer and primary and secondary antibodies are diluted in washing buffers, either phosphate buffered saline (PBS) or tris buffered saline containing Tween 20 (TBST). Afterwards, proteins in the membrane are usually detected with a secondary antibody linked to an enzyme such as horseradish peroxidase (HRP) and visualized by chemiluminescence detection. The use of chemiluminescence detection allows multiple film exposures and enables optimization of signal to noise. Chemiluminescent detection occurs when energy from a chemical reaction is released in the form of light. The most popular chemiluminescent western blotting substrates are luminol-based. For example, in the presence of horseradish peroxidase (HRP) and peroxide buffer, luminol oxidizes and forms an excited state product that emits light as it decays to the ground state. Light emission occurs only during the enzyme-substrate reaction; therefore, once the substrate in proximity to the enzyme is exhausted, signal output ceases. The two most common enzyme reporters that catalyze chemiluminescent reactions are HRP and alkaline phosphatase (AP). Enzyme-conjugated secondary antibodies are used for western blotting, and light-producing reactions are captured with X-ray film or with charge-coupled device (CCD) camera–based digital imaging instruments. The detection reagents can be removed and the entire blot reproved to visualize another protein or to optimize detection of the first protein. Finally, it should be emphasized that quantification of protein levels is always semi-quantitative because western blot data provides a relative comparison of protein levels, but not an absolute measure of quantity. The reason for the semiquantitative nature of western blot measurements is due to the variations in loading and transfer rates between the samples in separate lanes and to the fact that the signal generated by the detection is not linear across the concentration range of the samples [24]. However, the differences in protein loading are normally standardized, using and internal control or loading control protein which are proteins derived from ubiquitously expressed "housekeeping" genes and have been widely used due to their presumed

consistent level of expression across a diverse range of samples. Actin and tubulin are two of the most frequently used loading controls in biomedical research [26].

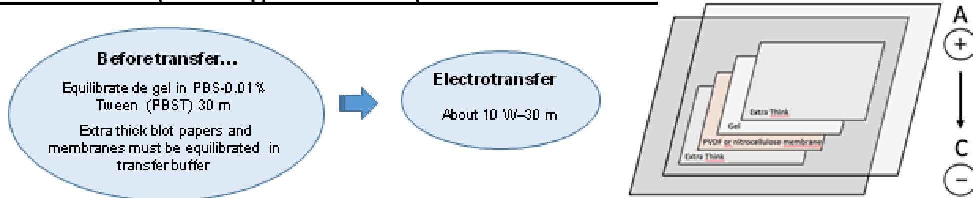
1. Preparation of lysates from cell cultures



2. Preparation of samples and electrophoresis: separate proteins by size



3. Electrotransfer (semi-dry): transfer the protein to membranes



4. Membrane blocking, washing, antibody incubation and chemiluminiscence detection

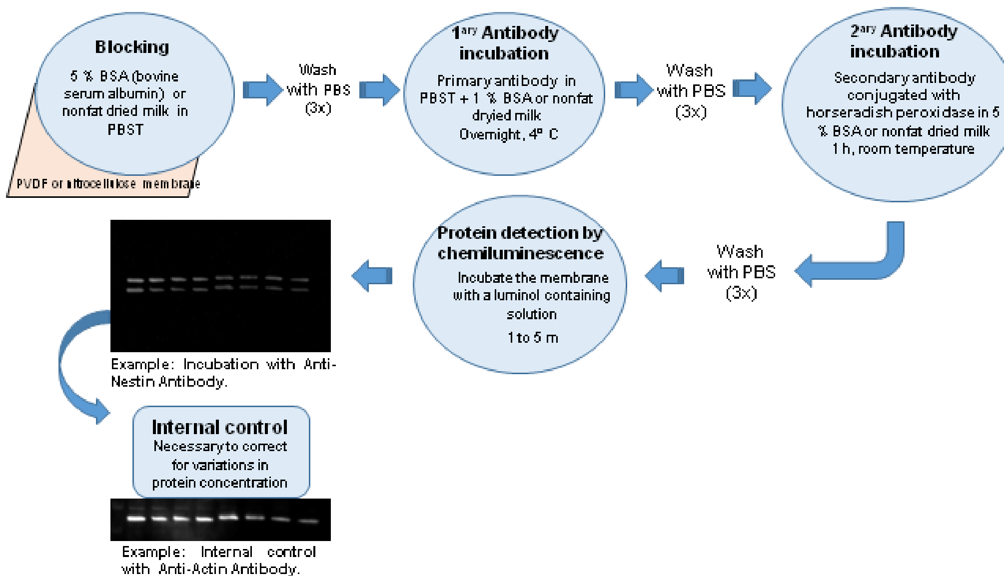


Figure 5.12. Schematic representation of a western blot protocol using cell lysates obtained from adherent cells.

HEL-30 Cytotoxicity Test: Determines the anabolic competence of the cell. HEL-30 cells are incubated in the presence of radiolabelled leucine with or without test chemical for a short period of time. Uptake of the radio-labeled leucine is terminated by the addition of unlabelled leucine. Cell protein is precipitated with trichloroacetic acid and harvested onto glass-fibre filters. The radioactivity of the samples is measured by liquid scintillation counting.

Human Lymphocyte Cytotoxicity Assay: measures the leakage of DNA and lactate dehydrogenase (LDH, EC. 1.1.1 27) from lymphocytes into the surrounding medium as an indicator of cytotoxicity and also includes a measure of mitochondrial activity trough the MTT assay. The method uses lymphocytes isolated from anticoagulated, normal, human blood samples and grown for 5 days, centrifuged and resuspended in complete medium, aliquoted into 24-well plates and exposed to the test chemicals at appropriate dilutions.

LS-L929 Cytotoxicity Test: this test uses L-929 fibroblasts (mouse) maintained in suspension culture and incubated in the presence of test material in a range of concentrations for 4 hours. Cell viability is then determined by uptake of the dyes ethidium bromide and fluorescein acetate. A resultant cytotoxic effect is quantified using two complementary fluorimetric assay procedures. After exposure to a test compound, viable cells may be identified by their ability to accumulate fluorescein on incubation with fluorescein diacetate (FDA). The nucleic acid of both viable and non-viable cells can be stained with ethidium bromide (EB). Non-viable cells (stained only with EB) may be distinguished from viable cells (stained with EB and with FDA) by the selective use of filters.

Laser Diffraction Measurement of Tumour Spheroids: Tumour cell lines cultured as aggregates can be utilized for in vitro radiosensitivity and/or chemosensitivity tests. Chemical effects are monitored by studying the changes in spheroid diameter measured by laser diffraction. Determines cell viability and morphology and has been used in human cervical colorectal and lung cancer cell lines.

Quantitative Video Microscopy of Intracellular Motion and Mitochondria-Specific Fluorescence: the test uses either IMR-90 fibroblasts (human) or L-

929 fibroblasts (mouse). IMR 90 cells are cultured on cover slips, mounted on slides and incubated for 1-24 hours in the presence or absence of test compounds. Movement of cell organelles is observed by means of video-enhanced contrast microscopy. The cells are maintained at a stable temperature and pH in an incubation chamber. At the same time the lysosomes and mitochondria are specifically stained with fluorescent vital dyes so that their number and morphology may be assessed. The analog video signal is enhanced, digitalized and subjected to several steps of image processing. The final images are recorded and later analyzed to provide plots of organelle velocity versus incubation time.

MTT Assay: The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) is taken up into cells and reduced in a mitochondria-dependent reaction to yield a formazan product [14, 16]. The product accumulates within the cell, due to the fact that it cannot pass through the plasma membrane. On solubilization of the cells, the product is liberated and can readily be detected and quantified by a simple colorimetric method. The method can be used almost with any cell type.

The ability of cells to reduce the MTT provides an indication of mitochondrial integrity and activity which, in turn, may be interpreted as a measure of viability and/or cell number. The test measures the tissue viability: determined by a reduction in mitochondrial dehydrogenase activity, measured by formazan salt production from MTT. The result is expressed as % of the negative control. The endpoint value is the tissue viability (%): calculated as the ratio (ODtreated)/(ODnegative control)×100%. Is useful to determine the TC_{50} or the IC_{50}: the concentration of a test substance that decreases the MTT reduction to formazan by 50%, determined from a dose-response curve.

The assay is useful to determine the viability of many cell types but not for cells with low mitochondrial activity. However, the number of cells initially plated, the period of exposure to chemicals, concentration of MTT, total duration of the experiment, etc. must be standardized for each cell line. The protocol used for the MTT assay in human neuronal CTX0E16 cells is summarized in Figure 5.13 and detailed bellow.

1. Cells seeded in 96 or 48 well plates are cultured for the desired time and treated with the compound of interest (in this case several DMSO concentrations: 0.001, 0.01, 0.1, 1, 2.5, 5 and 10 % DMSO v/v) and control wells are treated with the corresponding vehicle used to solubilize the compound and are incubated in the culture incubator for the desired time (in this case 24 hours in the incubator).
2. Remove the culture medium and wash three times with Locke´s buffer containing in mM: 154 NaCl, 5.6 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 5.6 glucose (pH 7.4). for 1 hour at 37 ºC.
3. Incubate the plates with 200 µl of 500 µg/ml MTT dissolved in Locke´s buffer per well, 60 min at 37 ºC. MTT solution should be prepared immediately before to the experiment, kept in the dark and discarded after using.
4. Carefully wash off excess MTT in order to avoid cell detachment and add 200 µl of 5% sodium dodecyl sulphate (SDS) al 5 %, 200 µl per well to disaggregate the cells and keep the plate overnight in the dark.
5. Transfer disaggregated cells from each well to 96 well plates and treat with 10 µl isopropanol to eliminate air bubbles.
6. Read the absorbance of the colored formazan salt at 595 nm in a spectrophotometer plate reader.

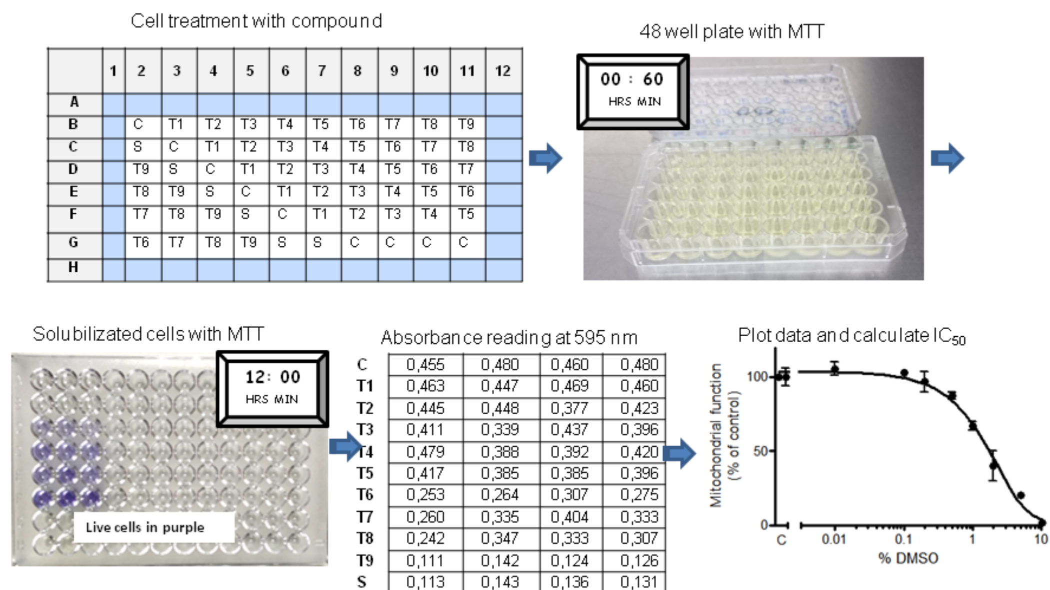


Figure 5.13. MTT test to measure cell viability in CTX0E16 human neuronal stem cells: Experimental design is shown in the left upper panel (in blue are empty wells): C = control cells, T = treated cells. Treatments: T1 = DMSO 0, 001 %, T2 = DMSO 0.01 %, T3 = DMSO 0,1 % , T4 = DMSO 0,2 %, T5 = DMSO 0,5 %, T6 = DMSO 1 %, T7 = DMSO 2 %, T8 = DMSO 5 %, T9 = DMSO 10 %; S = saponin (cell death control). Upper right panel: addition of MTT to treated cells. Lower left panel: MTT colored formazan salt in solubilized cells. Middle lower panel: 595 nm absorbance values reading with the corresponding treatment for one representative experiment. Right lower panel: Dose response plot showing the means of at least three independent experiments.

Resazurin (Alamar Blue™) Cytotoxicity Assay: this test is also used to determine the viability of cells in culture after exposure to chemicals. Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide, also called Alamar Blue™ by some authors) is a non-toxic, non-fluorescent blue dye which is reduced by metabolically active cells to the fluorescent red dye resorufin. This redox indicator is a widely used nontoxic reagent that exhibits both fluorimetric and colorimetric properties in response to metabolic activity. Resorufin has a pink color and is highly fluorescent. As the number of living cell decrease, the intensity of the fluorescence also decreases. The reduction-induced color

change varies proportionately with cell number and time, changing from a non-fluorescent color blue to a reduced pink fluorescent form [27]. A scheme of the procedure used to evaluate cytotoxicity using the resazurin assay is shown in Figure 5.14.

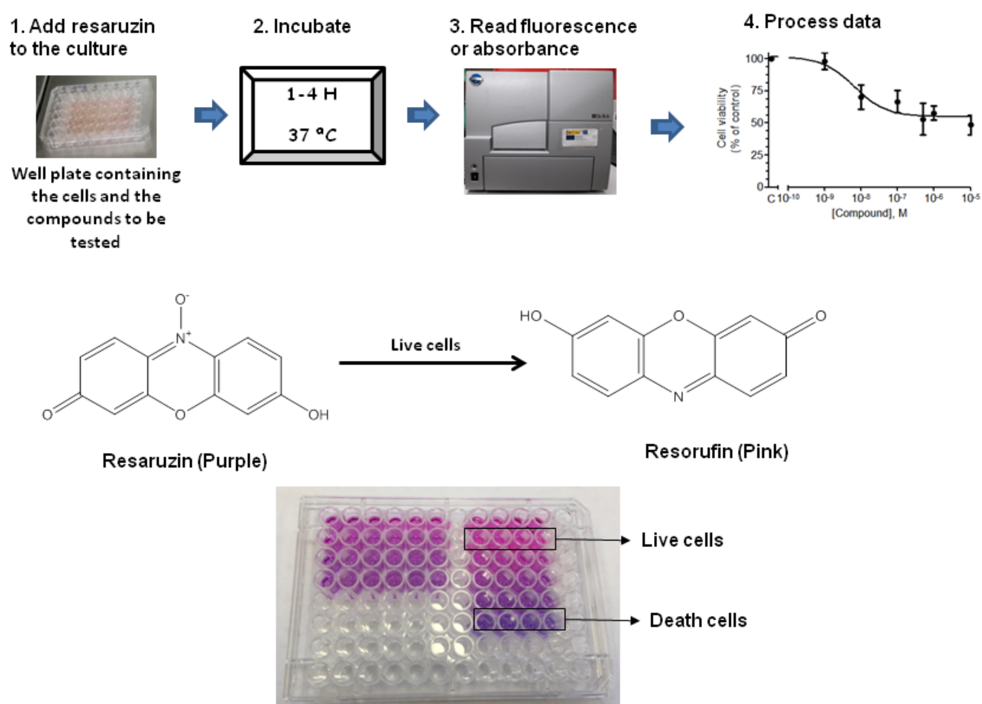


Figure 5.14. Scheme showing the protocol to evaluate cytotoxicity using the resazurin cytotoxicity assay and its mechanism of action.

Lactate dehydrogenase cytotoxicity assay: The measurement of lactate dehydrogenase (LDH) levels is a reliable tool to determine the toxicity of chemical compounds. The release of LDH to the cell culture media is an indicator of irreversible cell death resulting from the cell membrane damage [28, 29]. The LDH release can be measured through a two-step enzymatic reaction. Initially, LDH catalyses the conversion of lactate to pyruvate by reducing NAD^+ to NADH. Then, diaphorase utilizes NADH to reduce the tetrazolium salt, iodonitrotetrazolium chloride (INT), to produce a red formazan product. The quantity of formazan produced is directly correlated to the quantity of LDH

released to the medium. The level of formazan formation can be easily detected by measuring the absorbance at 490 nm. Absorbance should also be measured at 680 nm to detect the background absorbance value. The absorbance value obtained at 680 nm should be subtracted from the absorbance value measured at 490 nm to obtain the final absorbance value and, therefore, the quantification of formazan. Currently, there are multiple kits that allow us to measure the LDH release. Each assay kit brings specifications to follow, but in general, the schematic representation of LDH protocol along with the chemical reaction is represented in Figure 5.15.

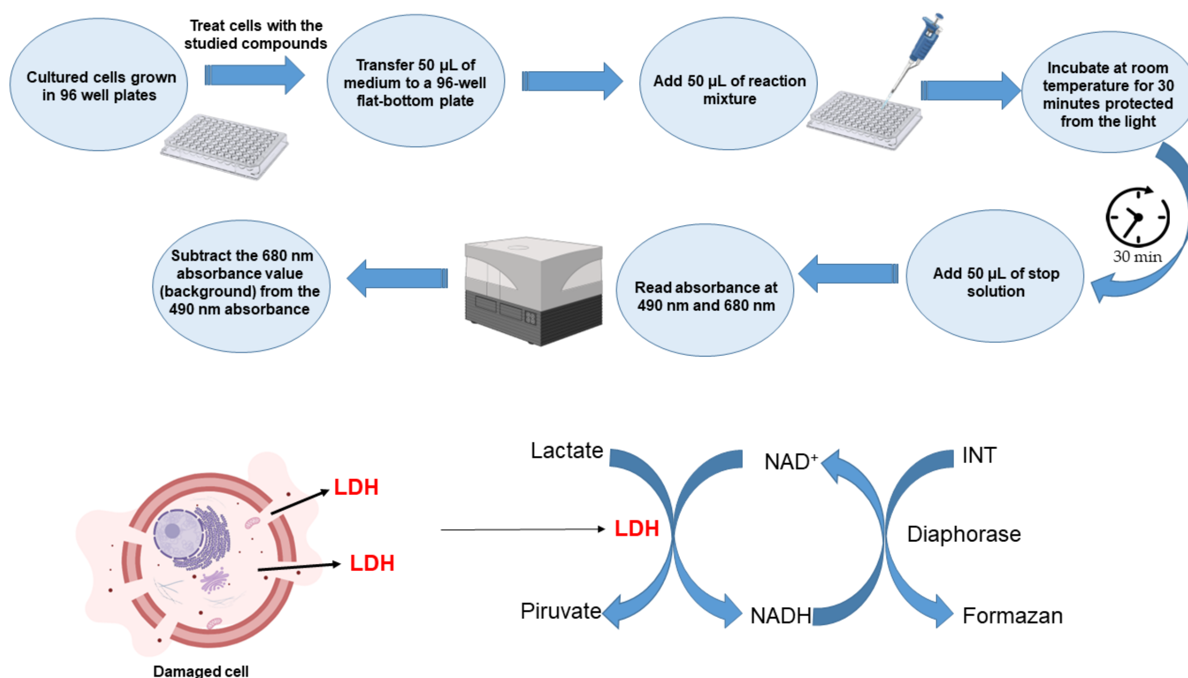


Figure 5.15: Schematic representation of the protocol to determine LDH release and the chemical reactions involved.

This method has many advantages as the reliability, speed, and the direct measurement of the LDH in the culture media, avoiding the need of wash cells. Therefore, being a reliable method of measuring cell death, in contrast to other assays to assess cell viability, such as the previously mentioned MTT study, where the number of cells at the beginning of the assay may not be the same as at the end since viable cells, but damaged ones can be removed by the

multiple washes that are needed. One of the major drawbacks of LDH assay is that the serum commonly used in culture media have an intrinsic LDH activity [30, 31]. Therefore, it is important to measure the absorbance obtained from the LDH activity in medium with the amount of serum used in the study. To reduce background signal, it is recommended to use the minimum serum percentage appropriate for each cell line without compromising cell viability.

Finally, in this section it should be mentioned that a draft guidance document on Good *In Vitro* Method Practices (GIVIMP) for the development and implementation of *in vitro* methods for regulatory use in human safety assessment has been coordinated by the European validation body EURL ECVAM and accepted on the work plan of the OECD test guideline program since April 2015 [5] and has been reviewed in 2017 [32]. This draft provides a list of several endpoints useful to evaluate cell toxicity, and the proposed tests are summarized in table 5.1.

Table 5.1. Summary of the different endpoints proposed to evaluate *in vitro* cytotoxicity as well as their main advantages and disadvantages. Adapted from [5].

Endpoint	Assay	Mechanism	Advantages/Disadvantages
1.Structural cell damage (non-invasive)	Evaluation of overall cell shape, cytoplasmic structure, flatness and outline properties on a good phase contrast light microscope	Screening assay	**Advantages:**, non-invasive **Disadvantages:** qualitative data, no exact cell death definition
	LDH-release test	Lactate dehydrogenase (LDH) enzyme is released to the culture medium when cell membranes rupture (non-viable cells), and the enzyme can then be measured in the supernatant.	**Advantages:** Measurement of a definite/unambiguous cell death endpoint; can be combined with cell function assays. Allows cells to be used for other purposes, if only supernatant is sampled. **Disadvantages:** Normalization necessary (extra wells for controls). Frequently high background LDH levels are observed (e.g. from serum components).
2. Structural cell damage (invasive)	Membrane penetration by dyes to detect 'cytotoxicity' (e.g. naphthalene black, trypan blue, propidium iodide, ethidium bromide, EH-	Involves the use of dyes that stain non-viable cells, but do not enter viable cells with an intact cell membrane.	**Advantages:** Rapid and usually easy to interpret. Gives information on the single cell level. **Disadvantages:** May overestimate viability since apoptotic cells continue to have intact membranes and may appear viable. Some dyes (e.g. trypan blue, H-33342) are cytotoxic, so that the evaluation has to be performed rapidly. Usually need to be combinated with dyes that stain both live and death

	1)		cells
	Retention of dyes within intact cells to detect 'viability' (e.g. fluorescein diacetate or calcein-AM)	The lipid-soluble dyes are transformed by cellular enzymes (esterases) into lipid-insoluble fluorescent compounds that cannot escape from cells with intact membranes.	**Advantages:** Rapid and usually easy to interpret. **Disadvantages:** Some cells leak the dyes and some dyes and some dyes can suffer photo-bleaching.
	Evaluation of programmed cell death/apoptosis markers	Activation of caspases (enzymatic analysis or staining) Activation or endonucleases (detectable as DNA-fragmentation). Chhromatin condensation (detectable by DNA stains) Detection of phosphatidylserine on the outside of the plasma membrane (annexin staining)	**Advantages:** Adds mechanistic information to cytotoxicity data. **Disadvantages:** Not all types of cell death may be detected by a given endpoint. Needs to be combined with a general cytotoxicity test. Some endpoints are prone to artifacts (annexin staining) and some staining techniques (TUNEL, caspase-3) lead to an un-intentional selection of subpopulations. Caspase activity measurement does not easily yield a prediction model for the extent of cell death.
3. Cell growth	Cell counting	For some cell populations impaired growth is considered as a reduction of viability.	**Advantages:** growth can be a sensitive parameter of cell well-being. **Disadvantages:** growth is not necessarily linked to cytotoxicity; artifacts Needs careful control in combination with cytotoxicity assays.
	BrdU or EdU incorporation	Measures new DNA synthesis based on incorporation of the easily detectable nucleoside analogs BrdU (or EdU) into DNA. .	**Advantages**: Measurement on single cell level. Easy to quantify. **Disadvantages**: BrdU/EdU can be cytotoxic; High cost and effort compared to counting.

	Staining of cellular components that are proportional to overall cell mass (proteins by e.g. sulforhodamine B or crystal violet; DNA by Hoechst H-33342)	These assays evaluate a surrogate measure of overall cell mass and assume that it correlates with total cell number	**Advantages**: Simple and cheap; lots of historical data **Disadvantages:** Mostly not a single cell measure but only population level. Protein staining is only a surrogate endpoint of real cell number. For DNA quantification with Hoechst 33342: fluorescent probe penetration, bleaching, and cytotoxicity are issues to be considered.
4. Cellular metabolism	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, or similar tetrazolium dye reduction assays	Measures the reduction of the tetrazolium dye by viable cells	**Advantages:** High throughput, easy, robust, low cost. Used in several ISO standards and OECD test guidelines. High sensitivity. Can be used for tissue constructs. **Disadvantages:** Measures amount of viable cells and needs control for contribution of proliferation. Cells with reduced mitochondrial function may appear non-viable. Measurement usually not on single cells.
	Resazurin (Alamar blue) reduction assay	Fluorescent resorufin is formed from resazurin through mitochondrial metabolism of viable cells.	**Advantages:** Many tests can be performed rapidly in multi-well dishes and cells can be tested repeatedly (non-invasive measurement). High sensitivity. **Disadvantages:** Cells with reduced mitochondrial function may appear non-viable. Some test items interfere with the assay (e.g. superoxide also reduces the dye)
	Mitochondrial depolarization assays (based on fluorescent indicator dyes)	Measurement of mitochondrial membrane potential by addition of potential sensing fluorescent dyes like JC-1, TMRE, MitoTracker, etc.	**Advantages**: fast, cheap, high throughput; single cell information. **Disadvantages**: as for MTT (measures cell function, not cytotoxicity). Artifacts by test items that affect mitochondria

			specifically. Artifacts by test items that affect plasma membrane potential, bleaching, quenching and unquenching, and shape changes and clustering of mitochondria.
	Neutral red assay (ISO 10993)	Active cells accumulate the red dye in lysosomes and the dye incorporation is measured by spectrophotometric analysis.	**Advantages:** Low cost. Used in several ISO standards and OECD test guidelines. **Disadvantages:** Normalization required for quantitative measurement, e.g. with protein content or number of cells. Gives usually information only at the population level. Not suited for tissue constructs and certain cell lines.
	ATP assays	Measurement of the total ATP content. Dying cells fail to produce ATP, have an increased ATP consumption, and may lose ATP through perforations of the plasma membrane. For the test, cell lysates are prepared, and the ATP content is assessed by a luminometric assay.	**Advantages:** fast, high throughput **Disadvantages:** no single cell data, expensive, not a direct measure of cytotoxicity.

IN VITRO METHODS TO TEST CELLULAR FUNCTION

To evaluate cellular function in living cells there are multiple techniques and several of them including cytosolic calcium determination, depolarization membrane detection and electrophysiological measurements are commonly used to evaluate the effects of marine compounds [12, 14-16, 33, 34].

Electrophysiology

Due to the complex equipment and specialized personnel required to perform electrophysiological determinations, next only the basic principles of electrophysiology are going to be summarized. In brief, electrophysiological recordings allow gaining insight on the effect of compounds on voltage-gated- and ligand-gated ion channels. The technique is based on the use of a recording pipette in narrow contact with the cell membrane (seal) and apply negative pressure in order to break the cell membrane and be able to measure either the current (in the modality of voltage-clamp) or the voltage (in the modality of current clamp) flowing through the cell membrane. A scheme showing some of the equipment required in an electrophysiology setup as well as the basis of the procedure to obtain access to the cell is shown in Figure 5.16.

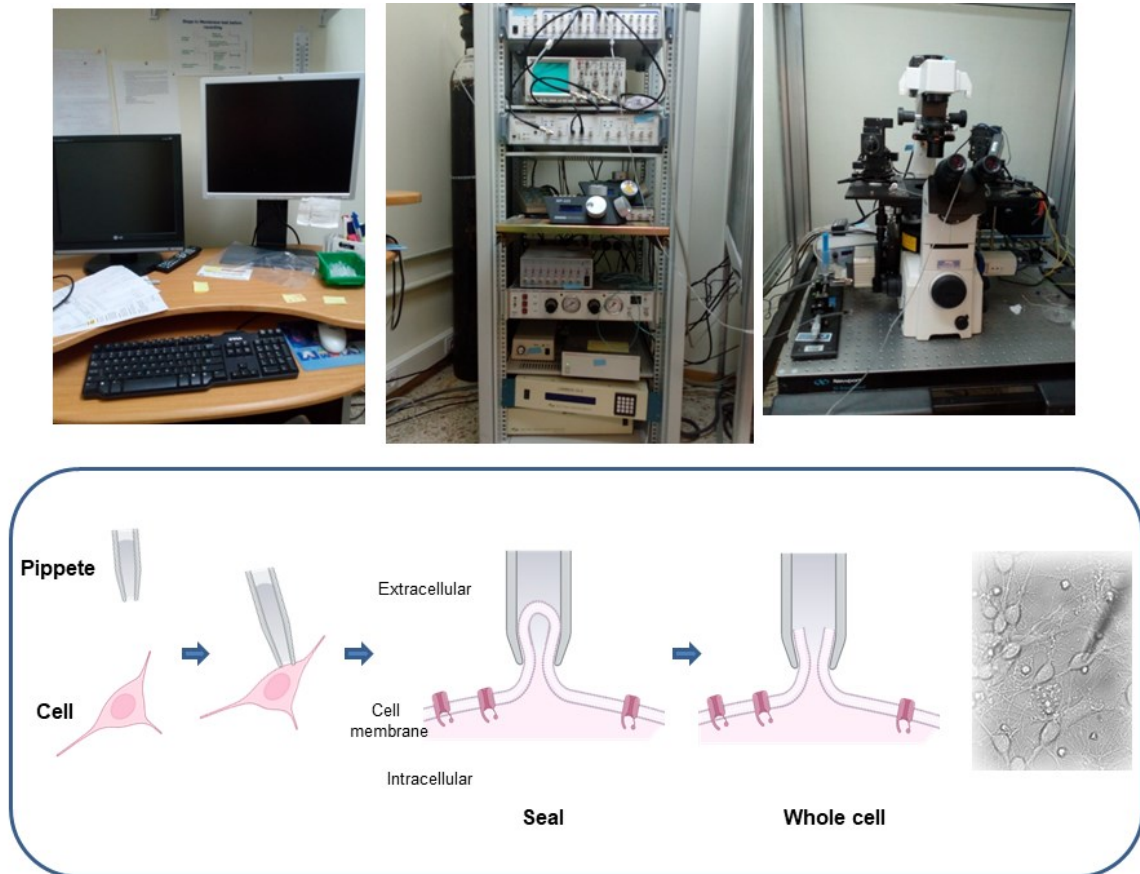


Figure 5.16. Electrophysiological equipment and schematic representation of the procedure to obtain a seal between the recording pipette and the cell.

Cytosolic calcium determination

Another powerful technique to assess cell function after chemical application is cytosolic calcium imaging. The increase in cytosolic calcium concentration has been shown to play an important role in vital cellular functions such as muscle contraction, cell secretion, oocyte fertilization, nerve conduction, embryo development and apoptosis in animals, plants and microbes, and in the invasion of mammalian cells by parasites, bacteria, and viruses. Therefore, live cell imaging of increases in cytosolic calcium concentration in cellular compartments has been investigated intensively. Multiple calcium imaging systems are now available commercially [35]. Among the first calcium indicators used for monitoring the dynamics of cellular calcium signaling were bioluminescent calcium-binding photoproteins, such as aequorin. However, for calcium imaging in living cells the most important achievement was the development of more sensitive and versatile fluorescent calcium indicators and buffers by Roger

Tsien and colleagues [36]. These indicators were the result of the hybridization of highly calcium-selective chelators like EGTA or BAPTA with a fluorescent chromophore. The first generation of fluorescent calcium indicators consisted of quin-2, fura-2, indo-1, and fluo-3. Quin-2 is excited by ultraviolet light (339nm) and was the first dye of this group to be used in biological experiments (see [37] for review). The widely used calcium sensitive fura-2 AM is a combination of calcium chelator and fluorophore. It is excitable by ultraviolet light (e.g., 350/380nm) and its emission peak is between 505 and 520 nm [36]. The binding of calcium ions causes intramolecular conformational changes that lead to a change in the emitted fluorescence. A summary illustrating the protocol for cytosolic calcium imaging in primary cultures of cerebellar granule cells is shown in Figure 5.17.

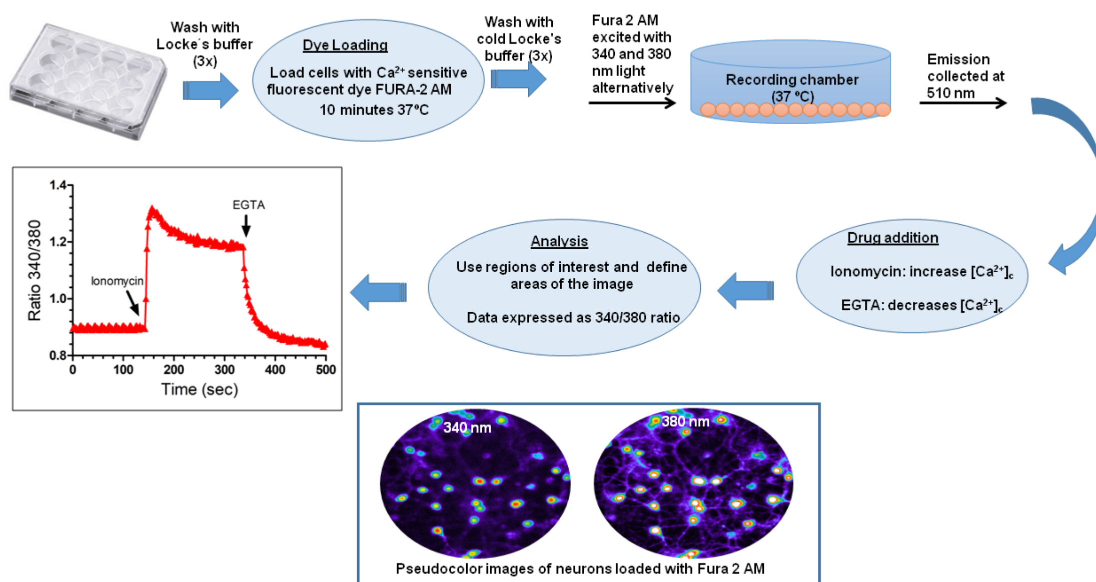


Figure 5.17. Schematic representation of the protocol for the determination of the cytosolic calcium concentration in primary cultures of cerebellar granule cells using the cell permeable calcium sensitive dye Fura-2 AM.

Membrane potential indicators

Membrane potential is an important physiological parameter of live cells with important different roles, especially in membrane-signalling processes as nerve-impulse propagation or muscle contraction [38]. In addition to the direct

measurement using electrophysiological techniques, there are also indirect membrane potential measurement techniques. These indirect measurements consist of ionic cellular dyes that stain the cell membrane and emit fluorescence. When membrane potential changes, the florescence emitted change as well, increasing with depolarization and decreasing with cell membrane hyperpolarization [39]. Therefore, by measuring the fluorescence emitted by cells after the exposure to a compound, it is possible to determine whether the compound has caused a change in membrane potential. Membrane potential indicators are classified in two groups, slow and fast responding dyes. The more conventional dye used is-(1,3-dibutylbarbituricacid) trimethine oxonol or $DiBAC_4(3)$. These dyes enter to depolarised cells and bind to intracellular proteins exhibiting an increase in fluorescence emitted. An increase in cell membrane depolarisation results in an increase of the influx of the dye. Therefore, the fluorescence signal gets more intense. Contrary to that, hyperpolarisation is detected by a decrease in fluorescence emission [40, 41]. There are a large number of commercial kits with the different membrane potential indicators that allows to detect changes in this parameter. Basing on the objective of the study, the most appropriate one will be chosen in each case. The detailed protocol for each dye varies. But in general, it consists on the measurement of the emitted fluorescence at the appropriate wavelength (excitation/emission) after cell exposure to the dye and the studied compound with the aim to detect either an increase or a decrease in fluorescence emission. General protocol and cellular basis are represented in Figure 5.18.

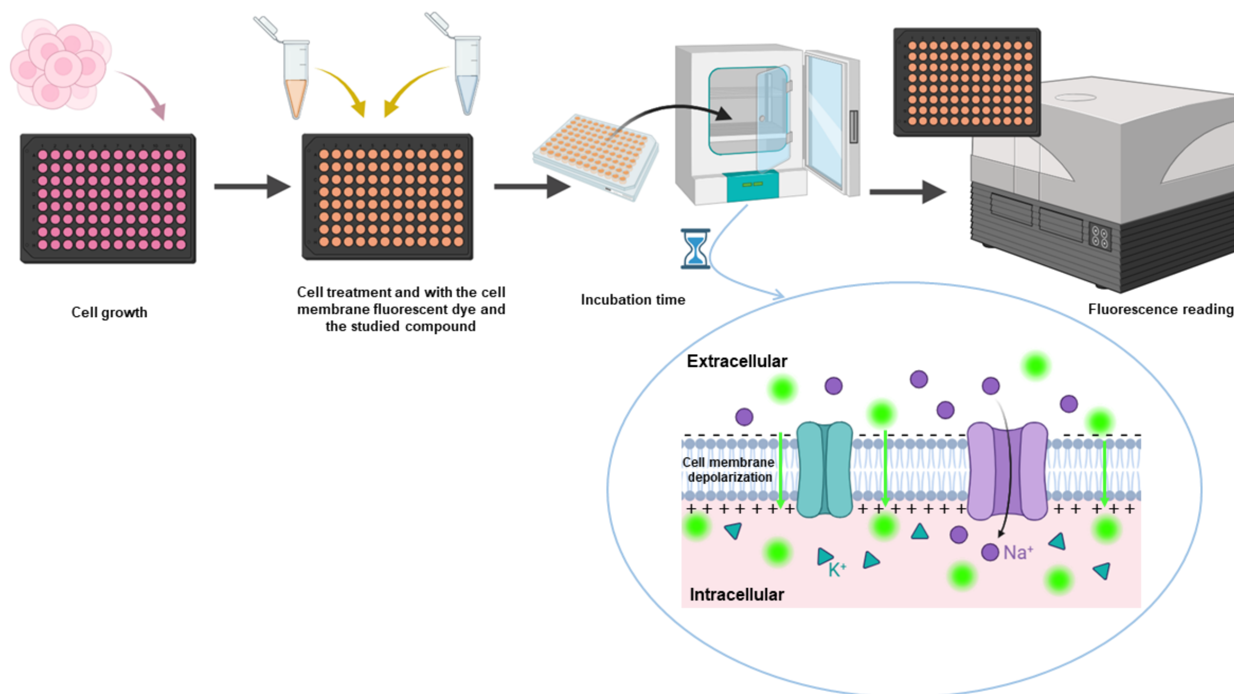


Figure 5.18: Schematic representation of the protocol to detect changes in membrane potential.

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