Chapter 1

In Vitro Cytotoxicity and Cell Viability Assays: Principles, Advantages, and Disadvantages

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.71923

Abstract

Cytotoxicity is one of the most important indicators for biological evaluation *in vitro* studies. *In vitro*, chemicals such as drugs and pesticides have different cytotoxicity mechanisms such as destruction of cell membranes, prevention of protein synthesis, irreversible binding to receptors etc. In order to determine the cell death caused by these damages, there is a need for cheap, reliable and reproducible short-term cytotoxicity and cell viability assays. Cytotoxicity and cell viability assays are based on various cell functions. A broad spectrum of cytotoxicity assays is currently used in the fields of toxicology and pharmacology. There are different classifications for these assays: (i) dye exclusion assays; (ii) colorimetric assays; (iii) fluorometric assays; and (iv) luminometric assays. Choosing the appropriate method among these assays is important for obtaining accurate and reliable results. When selecting the cytotoxicity and cell viability assays to be used in the study, different parameters have to be considered such as the availability in the laboratory where the study is to be performed, test compounds, detection mechanism, specificity, and sensitivity. In this chapter, information will be given about in vitro cytotoxicity and viability assays, these assays will be classified and their advantages and disadvantages will be emphasized. The aim of this chapter is to guide the researcher interested in this subject to select the appropriate assay for their study.

Keywords: cell viability, cytotoxicity, *in vitro* assays, advantages, disadvantages

1. Introduction

Viability levels and/or proliferation rates of cells are good indicators of cell health. Physical and chemical agents can affect cell health and metabolism. These agents may cause toxicity on cells via different mechanisms such as destruction of cell membranes, prevention of protein synthesis, irreversible binding to receptors, inhibition of polydeoxynucleotide

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elongation, and enzymatic reactions [1]. In order to determine the cell death caused by these mechanisms, there is a need for cheap, reliable and reproducible short-term cytotoxicity and cell viability assays.

In vitro cell viability and cytotoxicity assays with cultured cells are widely used for cytotoxicity tests of chemicals and for drug screening. Application of these assays has been of increasing interest over recent years. Currently, these assays are also used in oncological researches to evaluate both compound toxicity and tumor cell growth inhibition during drug development. Because, they are rapid, inexpensive and do not require the use of animals. Furthermore, they are useful for testing large number of samples. Cell viability and cytotoxicity assays are based on various cell functions such as cell membrane permeability, enzyme activity, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity [1].

In vitro cytotoxicity and/or cell viability assays have some advantages, such as speed, reduced cost and potential for automation, and tests using human cells may be more relevant than some in vivo animal tests. However, they have some disadvantages because they are not technically advanced enough yet, to replace animal tests [2].

It is important to know how many viable cells are remaining and/or how many cells are dead at the end of the experiment. A broad spectrum of cytotoxicity and cell viability assays is currently used in the fields of toxicology and pharmacology. The choice of assay method is crucial in the assessment of the interaction type [3].

2. Classification of cytotoxicity and cell viability assays

Although there are different classifications for cytotoxicity and cell viability assays, in this chapter, these assays are classified according to measurement types of end points (color changes, fluorescence, luminescent etc.).

1. Dye exclusion: Trypan blue, eosin, Congo red, erythrosine B assays.
2. Colorimetric assays: MTT assay, MTS assay, XTT assay, WST-1 assay, WST-8 assay, LDH assay, SRB assay, NRU assay and crystal violet assay.
3. Fluorometric assays: alamarBlue assay and CFDA-AM assay.
4. Luminometric assays: ATP assay and real-time viability assay.

2.1. Dye exclusion assays

The proportion of viable cells in a cell population can be estimated in various methods. The simplest and widely used one of the methods is dye exclusion method. In dye exclusion method, viable cells exclude dyes, but dead cells not exclude them. Although the staining procedure is quite simple, experimental procedure of large number of samples is difficult and time consuming [4]. Determination of membrane integrity is possible via dye exclusion

method. A variety of such dyes have been employed, including eosin, Congo red, erythrosine B, and trypan blue [5, 6]. Of the dyes listed, trypan blue has been used the most extensively [7–10].

If dye exclusion assays are used, following factors must be considered (i) lethally damaged cells by cytotoxic agents may require several days to lose their membrane integrity, (ii) the surviving cells may continue to proliferate during this time, and (iii) some lethally damaged cells are not appear to be stained with dye at the end of the culture period, because they may undergo an early disintegration. Factors (ii) and (iii) may cause an underestimate of cell death when the results of the assay are based on percent viability expression [11–13].

Dye exclusion assays have unique advantages for chemosensitivity testing. They are comparatively simple, require small numbers of cells, are rapid, and are capable of detecting cell kill in nondividing cell populations. Further investigations into the possible role of these assays in chemosensitivity testing are warranted [11]. However, none of these dyes is recommended for use on monolayer cell cultures but rather they are intended for cells in suspension; thus monolayer cells must first trypsinized [6].

2.1.1. Trypan blue dye exclusion assay

This dye exclusion assay is used to determine the number of viable and/or dead cells in a cell suspension. Trypan blue is a large negatively charged molecule. Trypan blue dye exclusion assay is based on the principle that live cells possess intact cell membranes that exclude this dye, whereas dead cells do not. In this assay, adherent or nonadherent cells are incubated with serial dilutions of test compounds for various times. After the compound treatment, cells are washed and suspended. Cell suspension is mixed with dye and then visually examined to determine whether cells take up or exclude dye. Viable cells will have a clear cytoplasm, whereas dead cells will have a blue cytoplasm [14, 15]. Number of viable and/or dead cells per unit volume is determined by light microscopy as a percentage of untreated control cells [15, 16].

Advantages: This method is simple, inexpensive, and a good indicator of membrane integrity [17], and dead cells are colored blue within seconds of exposure to the dye [18].

Disadvantages: Cell counting is generally done using a hemacytometer [19]. Therefore, counting errors (~10%) could be occurred. Counting errors have been attributed to poor dispersion of cells, cell loss during cell dispersion, inaccurate dilution of cells, improper filling of the chamber and presence of air bubbles in the chamber [17].

While the staining procedure is quite simple, it is difficult to process large number of samples concurrently, particularly where the exact timing of progressive cytotoxic effects is required [4]. Furthermore, trypan blue staining cannot be used to distinguish between the healthy cells and the cells that are alive but losing cell functions. Therefore, it is not sufficiently sensitive to use for in vitro cytotoxicity testing. Another disadvantage of trypan blue is toxic side effect of this dye on mammalian cells [20].

2.1.2. Erythrosine B dye exclusion assay

Erythrosine B, also known as erythrosine or Red No. 3, is primarily used as food coloring agent [20, 21]. Erythrosine B has already been introduced as a vital dye for counting viable cells. Principle of this dye exclusion assay is similar to trypan blue dye exclusion assay principle. Although erythrosine B is an alternative bio-safe vital dye for cell counting; it is not widely used to count viable or dead cells.

Advantages: It has benefits such as low cost, versatility, and bio-safety [20].

Disadvantages: Its procedure is time-consuming and labor-intensive. Moreover, potential disadvantages include contamination of reusable cell counting chamber, variations of hemocytometer filling rates, and inter-user variations [20].

2.2. Colorimetric assays

Principle of colorimetric assays is the measurement of a biochemical marker to evaluate metabolic activity of the cells. Reagents used in colorimetric assays develop a color in response to the viability of cells, allowing the colorimetric measurement of cell viability via spectrophotometer. Colorimetric assays are applicable for adherent or suspended cell lines, easy to perform, and comparably economical [22, 23]. Commercial kits of colorimetric assays are available from several companies and generally experimental procedures of these assays are available in kit packages.

2.2.1. MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide) assay is one of the most commonly used colorimeteric assay to assess cytotoxicity or cell viability [24]. This assay determines principally cell viability through determination of mitochondrial function of cells by measuring activity of mitochondrial enzymes such as succinate dehydrogenase [18]. In this assay, MTT is reduced to a purple formazan by NADH. This product can be quantified by light absorbance at a specific wavelength.

Advantages: This method is far superior to the previously mentioned dye exclusion methods because it is easy to use, safe, has a high reproducibility, and is widely used to determine both cell viability and cytotoxicity tests [18, 25].

Disadvantages: MTT formazan is insoluble in water, and it forms purple needle-shaped crystals in the cells. Therefore, prior to measuring the absorbance, an organic solvent such as dimethyl sulfoxide (DMSO) or isopropanol is required to solubilize the crystals. Additionally, the cytotoxicity of MTT formazan makes it difficult to remove cell culture media from the plate wells due to floating cells with MTT formazan needles, giving significant well-to-well error [18, 26].

Additional control experiments should be conducted to reduce false-positive or false-negative results that caused by background interference due to inclusion of particles. This interference could lead to an overestimation of the cell viability. This can often be controlled by subtraction of the background absorbance of the cells in the presence of the particles, but without the assay reagents [18, 26].

2.2.2. MTS assay

The MTS assay (5-(3-carboxymethoxyphenyl)-2-(4,5-dimethyl-thiazoly)-3-(4-sulfophenyl) tetrazolium, inner salt assay) is a colorimetric assay. This assay is based on the conversion of a tetrazolium salt into a colored formazan by mitochondrial activity of living cells. The amount of produced formazan is depend on the viable cell number in culture and can be measured with spectrophotometer at 492 nm.

Advantages: Previous studies suggest that the MTS in vitro cytotoxicity assay combines all features of a good measurement system in terms of ease of use, precision, and rapid indication of toxicity [27, 28]. MTS assay is a rapid, sensitive, economic, and specific in vitro cytotoxicity assay. Performance of this assay is very competitive to other toxicological tests. This assay provides ideal properties for cytotoxicity measurement because it is easy to use, rapid, reliable, and inexpensive. Therefore, it can be used for onsite toxicological assessments [27, 29–31].

Disadvantages: The level of absorbance measured at 492 nm is influenced by the incubation time, cell type, and cell number. The proportion of MTS detection reagents to cells in culture also influences the measured absorbance level. Previous studies suggested a linear relationship between incubation time and absorbance for short incubation times up to 5 hours [29, 32, 33]. Therefore, proper incubation times for this assay are 1–3 hours.

2.2.3. XTT assay

A colorimetric method based on the tetrazolium salt XTT (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-carboxanilide-2H-tetrazolium, monosodium salt) was first described by Scudiero et al. [34]. While MTT produced a water-insoluble formazan compound which required dissolving the dye in order to measure its absorbance, the XTT produces a water-soluble dye. The procedure of XTT is simply for measuring proliferation and is therefore an excellent solution for quantitating cells and determining their viability. XTT is used to assay cell proliferation as response to different growth factors. It is also used for assaying cytotoxicity.

This assay is based on the ability reduction of the tetrazolium salt XTT to orange-colored formazan compounds by metabolic active cells. Orange-colored formazan is water soluble and its intensity can be measured with a spectrophotometer. There is a linear relationship between the intensity of the formazan and the number of viable cells. The use of multiwell plates and a spectrophotometer (or ELISA reader) allows for study with a large number of samples and obtaining results easily and rapidly. The procedure of this assay includes cell cultivation in a 96-well plate, adding the XTT reagent and incubation for 2–24 hours. During the incubation time, an orange color is formed and the intensity of color can be measured with a spectrophotometer [34, 35].

Advantages: XTT assay is speed, sensitive, easy to use, and safe method. It has high sensitivity and accuracy [35].

Disadvantages: XTT assay performance depends on reductive capacity of viable cells with the mitochondrial dehydrogenase activity. Therefore, changes of reductive capacity of viable cells resulting from enzymatic regulation, pH, cellular ion concentration (e.g., sodium, calcium, potassium), cell cycle variation, or other environmental factors may affect the final absorbance reading [34, 35].

2.2.4. WST-1 assay

WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H* tetrazolium monosodium salt) cell proliferation assay is a simple, colorimetric assay designed to measure the relative proliferation rates of cells in culture. The principle of this assay is based on the conversion of the tetrazolium salt WST-1 into a highly water-soluble formazan by mitochondrial dehydrogenase enzymes in the presence of intermediate electron acceptor, such as mPMS (1-methoxy-5-methyl-phenazinium methyl sulfate) [36]. The water-soluble salt is released into the cell culture medium. Within incubation period, the reaction produces a color change which is directly proportional to the amount of mitochondrial dehydrogenase in cell culture and thus, the assay measures the metabolic activity of cells.

To perform the assay, the WST-1 reagent that is ready-to-use is added directly into the media of cells cultured in multiwell plates. The cultures are then given 30 minutes–4 hours to reduce the reagent into the dye form. The plate is then immediately read at 450 nm with a reference reading at 630 nm [37].

Advantages: It is easy to use, safe, has a high reproducibility, and is widely used to determine both cell viability and cytotoxicity tests. Furthermore, phenol red indicators in cell culture medium do not interfere with the dye reaction. Because the colored dye which produced at the end of experiment is water-soluble, it is not required a solvent and additional incubation time [37].

Disadvantages: The standard incubation time of WST-1 time is 2 h. Whether one-time addition of WST-1 can reflect the effect of the testing agents at different time points on the trend of relative cell viability is still unclear [37].

2.2.5. WST-8 assay

WST-8 assay is a colorimetric assay for the determination of viable cell numbers and can be used for cell proliferation assays as well as cytotoxicity assays. WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H* tetrazolium, monosodium salt), a highly stable and water-soluble WST, is utilized in Cell Counting Kit-8 (CCK-8). It is more sensitive than WST-1 particularly at neutral pH [37]. Because of the electron mediator, 1-methoxy PMS in this kit is highly stable, and CCK-8 is stable for at least 6 months at the room temperature and for 1 year at 0–5°C. Since WST-8, WST-8 formazan, and 1-methoxy PMS have no cytotoxicity on cells in the culture media, same cells from the previous assay may be used for additional experiments.

Advantages: WST-8 is not cell permeable, which results in low cytotoxicity. Therefore, after the assay, it is possible to continue further experiments using the same cells. Furthermore, it produces the water-soluble formazan upon cellular reduction, which would provide an additional advantage to the method by allowing a simpler assay procedure and not required an extra step to dissolve the formazan [28].

Disadvantages: An important consideration is that reduction of assay substrates is impacted by changes in intracellular metabolic activity that has no direct effect on overall cell viability [15].

2.2.6. LDH (lactate dehydrogenase) assay

LDH (lactate dehydrogenase) cytotoxicity assay is a colorimetric method of assaying cellular cytotoxicity. LDH Cytotoxicity Assay Kit can be used with different cell types not only for assaying cell-mediated cytotoxicity but also for assessment of cytotoxicity mediated by toxic chemicals and other test compounds. The assay measures the stable, cytosolic, lactate dehydrogenase (LDH) enzyme quantitatively. This enzyme releases from damaged cells. LDH is an enzyme that is normally found within the cell cytoplasm. When cell viability reduced leakiness of the plasma membrane increase and therefore LDH enzyme is released into the cell culture medium. The released LDH is measured with a coupled enzymatic reaction that results in the conversion of a tetrazolium salt (iodonitrotetrazolium (INT)) into a red color formazan by diaphorase. In the first step, LDH catalyze conversion of lactate to pyruvate and thus NAD is reduced to $NADH/H^+$. In a second step, catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), which is reduced to red formazan [38, 39].

The LDH activity is determined as NADH oxidation or INT reduction over a defined time period. The resulting red formazan absorbs maximally at 492 nm and can be measured quantitatively at 490 nm.

The detergent Triton X-100 is commonly used as positive control in the LDH assay to determine the maximum LDH release from the cells. In addition, well-known membranolytic particles such as crystalline silica can be used as a positive control in LDH assay [40].

Advantages: Reliability, speed, and simple evaluation are some of characteristics of this assay. Because, the loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage [38, 41].

Disadvantages: The major limitation of this assay is that serum and some other compounds have inherent LDH activity. For example, the fetal calf serum has extremely high background readings. Therefore, this assay is limited to serum-free or low-serum conditions, limiting the assay culture period (depending on your cells' tolerance to low serum) and reducing the scope of the assay as it can no longer allow determination of cell death caused under normal growth conditions (i.e. in 10% fetal calf serum). At a minimum, you should always first test the assay with an unused aliquot of the media you intend to use and compare the reading to that from media lacking supplements (e.g. straight DMEM) [42].

2.2.7. SRB (Sulforhodamine B) assay

SRB (Sulforhodamine B) assay is a rapid and sensitive colorimetric method for measuring the drug-induced cytotoxicity in both attached and suspension cell cultures. This assay as first described by Skehan and colleagues was developed for use in the disease-orientated, large-scale anticancer drug discovery program of the National Cancer Institute (NCI) that was launched in 1985. SRB is a bright pink aminoxanthene dye with two sulfonic groups. Under mildly acidic conditions, SRB binds to protein basic amino acid residues in TCA-fixed (trichloroacetic acid) cells to provide a sensitive index of cellular protein. SRB assay is also used to evaluate colony formation and colony extinction [43].

Advantages: The SRB assay is simple, fast, and sensitive. It provided good linearity with cell number, permitted the use of saturating dye concentrations, is less sensitive to environmental fluctuations, is independent of intermediary metabolism, and provided a fixed end point that is not require a time-sensitive measurement of initial reaction velocity [43]. Reproducibility of this assay is high.

Disadvantages: It is important to obtain and maintain a homogeneous cell suspension. Cellular clumps/aggregates should be avoided for high assay performance.

2.2.8. NRU (neutral red uptake) assay

The neutral red uptake (NRU) assay is also one of the most used colorimetric cytotoxicity/cell viability assay. This assay was developed by Borenfreund and Puerner [44]. This assay was based on the ability of viable cells to take up the supravital dye neutral red. This weakly cationic dye penetrates cell membranes by nonionic passive diffusion and concentrates in the lysosomes. The dye is then extracted from the viable cells using an acidified ethanol solution and the absorbance of the dye is measured using spectrophotometer.

Neutral red uptake depends on the capacity of cells to maintain pH gradients through the ATP production. At physiological pH, net charge of the dye is zero. This charge enables the dye to penetrate the cell membranes. Inside the lysosomes, there is a proton gradient to maintain a pH lower than that of the cytoplasm. Thus, the dye becomes charged and is retained inside the lysosomes. When the cell dies or pH gradient is reduced, the dye cannot be retained. In addition, the uptake of neutral red by viable cells can be modified by alterations in cell surface or lysosomal membranes. Thus, it is possible to distinguish between viable, damaged, or dead cells [44]. Lysosomal uptake of neutral red dye is a highly sensitive indicator of cell viability. The assay can quantitate cell viability and measure cell replication, cytostatic effects or cytotoxic effects depending on the seeding density [45]. Absorbance is measured at 540 nm in multiwell plate reader spectrophotometer.

Advantages: NRR assay is a good marker of lysosomal damage. Also, speed and simple evaluation are some advantages of this assay.

Disadvantages: It has been reported that the NRR assay is either minimally or not at all affected by natural factors, such as temperature and salinity, but is mainly influenced by pollutants [46].

2.2.9. CVS assay (crystal violet assay)

Adherent cells detach from cell culture plates during cell death. This feature can be used for the indirect assessment of cell death and to determine differences in proliferation rate upon stimulation with cytotoxic agents. One simple method to detect maintained adherence of cells is crystal violet assay. In this assay, crystal violet dye binds to proteins and DNA of viable cells, and thus, attached cells are stained with this dye. Cells lose their adherence during cell death and are subsequently lost from the population of cells, reducing the amount of crystal violet staining in a culture. Crystal violet assay is a quick and reliable screening method that is suitable for the examination of the impact of chemotherapeutics or other compounds on cell survival and growth inhibition [47].

Advantages: Crystal violet staining is a quick and versatile assay for screening cell viability under diverse stimulation conditions [48]. However, it is potentially compromised by proliferative responses that occur at the same time as cell death responses. Therefore, chemical inhibitors of caspases and/or of necroptosis may be incorporated into the assay [49, 50]. Alternatively, molecular studies (e.g., overexpression or knockdown) can be performed to more specifically address the nature of cell death [51].

Disadvantages: Crystal violet assay is insensitive to changes in cell metabolic activity. Therefore, this assay is not appropriate for studies used cell metabolism affected compounds. While crystal violet assay is suitable for the examination of the impact of chemotherapeutics or other compounds on cell survival and growth inhibition, it is not able to measure cell proliferation rate [51].

2.3. Fluorometric assays

Fluorometric assays of cell viability and cytotoxicity are easy to perform with the use of a fluorescence microscope, fluorometer, fluorescence microplate reader or flow cytometer, and they offer many advantages over traditional dye exclusion and colorimetric assays. Fluorometric assays are also applicable for adherent or suspended cell lines and easy to use. These assays are more sensitive than colorimetric assays [52–54]. Commercial kits of fluorometric assays are available from several companies and generally experimental procedures of these assays are available in kit packages.

2.3.1. alamarBlue (AB) assay

alamarBlue assay is also known as resazurin reduction assay. The alamarBlue assay is based on the conversion of the blue nonfluorescent dye resazurin, which is converted to the pink fluorescent resorufin by mitochondrial and other enzymes such as diaphorases [53].

Resazurin is a phenoxazin-3-one dye and cell permeable redox indicator that can be used to monitor viable cell number with protocols similar to those utilizing the tetrazolium compounds [55]. It is known to act as an intermediate electron acceptor in the electron transport chain between the final reduction of oxygen and cytochrome oxidase by substituting for molecular oxygen as an electron acceptor [52]. It is a nontoxic and cell permeable compound. Color of this compound is blue and it is nonfluorescent. After entering cells, resazurin is reduced to resorufin. Resorufin is red in color and highly fluorescent compound. Viable cells convert continuously resazurin to resofurin, increasing overall fluorescence and color of the cell culture medium. The quantity of produced resofurin is related to the number of viable cells. Ratio of viable cells can be quantified using a microplate reader fluorometer equipped with a 560 nm excitation/590 nm emission filter set. Resofurin can also be measured by absorbance changes, but absorbance detection is not often used because absorbance detection is less sensitive than fluorescence measurement.

The incubation period required to generate a sufficient fluorescent signal above background is usually about 1–4 hours, depending on metabolic activity of the cells, the cell density per well and other conditions such as the culture medium type [54].

Advantages: alamarBlue (resazurin reduction) assay is relatively inexpensive and more sensitive than tetrazolium assays. Also, it can be multiplexed with other methods such as measuring caspase activity to gather more information about the cytotoxicity mechanism.

Disadvantages: Fluorescent interference from test compounds and the often overlooked direct toxic effects on the cells are possible [54].

2.3.2. CFDA-AM assay

CFDA-AM (5-carboxyfluorescein diacetate, acetoxymethyl ester) is another fluorogenic dye that is used for cytotoxicity determination. It is indicator for plasma membrane integrity. The dye CFDA-AM is nontoxic esterase substrate that can be converted by nonspecific esterases of viable cells from a membrane permeable, nonpolar, nonfluorescent substance to polar, fluorescent dye, carboxyfluorescein (CF). The conversion of CFDA-AM to CF by the cells indicates the integrity of plasma membrane, since only an intact membrane can maintain the cytoplasmic milieu which is needed to support esterase activity [56].

Advantages: CFDA-AM and alamarBlue assays were shown to be applicable in parallel on the same set of the cells, since both are nontoxic to cells, require similar incubation times, and can be detected at different wavelengths without interferences [56–58].

Disadvantages: Fluorescent interference from test compounds is possible.

2.3.3. Protease viability marker assay (GF-AFC assay)

Measurement of a conserved and constitutive protease enzyme activity of viable cells is used as a good indicator of cell viability. A cell permeable fluorogenic protease substrate (glycylphenylalanyl-aminofluorocoumarin; GF-AFC) has been recently developed to selectively detect protease activity that is restricted to viable cells [59]. The GF-AFC substrate can penetrate viable cells. In these cells, cytoplasmic aminopeptidase activity removes the gly and phe amino acids to release aminofluorocoumarin (AFC) and produce a fluorescent signal proportional to the number of viable cells [54].

When cells die, this protease activity is rapidly loss. Therefore, this protease activity is a selective marker of the viable cell population. The signal generated from this assay approach has been shown to correlate well with other established methods of determining cell viability such as an ATP assay [54].

Advantages: It is relatively nontoxic to cells in culture. Also, in opposite to exposure of cells to tetrazolium, long-term exposure of the GF-AFC substrate cells results in little change in viability of cells. This assay is suitable for multiplexing with other assays, because at the end of the assay, cell population remains viable and can be used for subsequent assays. Furthermore, the incubation time is much shorter (30 min-1 hour) compared to 1–4 hours required for the tetrazolium assays [54].

Disadvantages: Fluorescent interference from test compounds is possible.

2.4. Luminometric assays

Luminometric assays provide fast and simple determination of cell proliferation and cytotoxicity in mammalian cells. These assays can be performed in a convenient 96-well and 384-well microtiter plate format and detection by luminometric microplate reader [54, 60, 61]. A remarkable feature of the luminometric assays is the persistent and stable glow-type signal produced after reagent addition. This attribute can be harnessed to produce both viability and cytotoxicity values from the same well [59]. Commercial kits of luminometric assays are available from several companies and generally experimental procedures of these assays are available in kit packages.

2.4.1. ATP assay

ATP (adenosine tri-phosphate) represents the most important chemical energy reservoir in cells and is used for biological synthesis, signaling, transport, and movement processes. Therefore, cellular ATP is one of the most sensitive end points in measuring cell viability [62]. When cells damaged lethally and lose membrane integrity, they lose the ability to synthetize ATP and the ATP level of cells decreases dramatically [54, 63]. The ATP assay is based on the reaction of luciferin to oxyluciferin. Enzyme luciferase catalyzes this reaction in the presence of Mg^{2+} ions and ATP yielding a luminescent signal. There is a linear relationship between the intensity of luminescent signal and ATP concentration [61] or cell number [64].

The ATP assay chemistry can typically detect fewer than 10 cells per well, and therefore, it has been widely used 1536-well plate format.

Advantages: ATP assay is the fastest cell viability assay to use, the most sensitive, and is less prone to artifacts than other viability assays. The luminescent signal reaches steady state and stabilizes within 10 min after addition of reagent. It does not have an incubation step for conversion of substrate into colored compound. This also eliminates a plate handling step [54].

Disadvantages: The ATP assay sensitivity is usually limited by reproducibility of pipetting replicate samples rather than a result of the assay chemistry [54].

2.4.2. Real-time viability assay

Recently, a new approach is developed to measure viable cell number in real time [60]. In this assay, an engineered luciferase derived from a marine shrimp and a small molecule prosubstrate is used. The pro-substrate and luciferase are added directly to the cell culture medium as a reagent. The pro-substrate is not a substrate of luciferase. Viable cells with an active metabolism reduce the pro-substrate into a substrate, which used by luciferase, to generate a luminescent signal. The assay can be performed in two formats: continuous read and endpoint measurement. In the continuous read format, the luminescent signal can be repeatedly recorded from the sample wells over an extended period to measure the number of cells in "real time" [54, 60].

Advantages: This assay is the only assay which allows to real-time measurement of cell viability/cytotoxicity. The rapid decrease in luminescent signal following cell death enables multiplexing this assay with other luminescent assays that contain a lysis step that will kill cells. The decrease in luminescence following cell death is important to eliminate interference with subsequent luminescent assays [54, 60].

Disadvantages: A limitation of the real time assay results from the eventual depletion of pro-substrate by metabolically active cells. Generally, the luminescent signal generated correlates with the number of metabolically active cells. However, the length of the time the luminescent signal will be linear with cell number will depend on the number of cells per well and their metabolic activity. Therefore, it is recommended that the maximum incubation time to maintain linearity should be empirically determined for each cell type and seeding density [54, 60].

3. Conclusions

A broad spectrum of cytotoxicity and cell viability assays is currently used in the fields of toxicology and pharmacology. An ideal assay for *in vitro* viability and/or cytotoxicity determination should be a rapid, safe, reliable, efficient, and time- and cost-effective. It should not interfere with test compound. The choice of assay method is crucial in the assessment of the interaction type. The assay may change the interpretation of the compound interaction. Therefore, the assay method should be chosen with caution, considering the mechanism of action of the test compound [3]. Tissue or cell type used in the study also affects the performance of cytotoxicity and/or cell viability assays. Therefore, before choosing an assay for study, different methods should be tried and compared. If it is possible, more than one assay should be used to determine cytotoxicity and/or cell viability in *in vitro* studies. Thus, reliability of the obtained results would increase.

Acknowledgements

The author would like to thank Asst. Prof. Tülay AŞKIN ÇELİK for her helpful advice in this chapter.

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