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GUIDELINE

Guidelines for cell viability assays

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Abstract

Recently, the interest in the application of cell viability assays has been increasing in various fields. Cell viability assays may be broadly classified as (a) dye exclusion assays, (b) colorimetric assays, (c) fluorometric assays, (d) luminometric assays, and (e) flow cytometric assays. Dye exclusion assays include trypan blue, eosin, congo red, and erythrosine B stain assays, whereas 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt (WST-1), 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8), lactate dehydrogenase (LDH), sulforhodamine B (SRB), neutral red uptake (NRU), and crystal violet stain (CVS) assays are among the colorimetric assays. Similarly, resazurin and 5-carboxyfluorescein diacetate acetoxymethyl ester (5-CFDA-AM) assays are based on fluorometric measurements, whereas luminometric assays comprise adenosine triphosphate and real-time viability assays. Major flow cytometric assays include membrane asymmetry, membrane permeability, and mitochondria assays. In this guideline, the mechanisms and the practice of assessment of the most common cell viability assays applied in research labs are discussed in detail. An ideal cell viability assay should be safe, rapid, reliable, efficient, and time- and cost-effective, and should not interfere with the test compound. Overall, it can be concluded that more than one cell viability assay should be applied in order to obtain reliable results.

KEYWORDS
annexin V staining, ATP assay, MTT assay, resazurin assay, trypan blue stain assay

1 | INTRODUCTION

Cell viability is defined as the number of healthy cells in a sample. The measurement of cell viability plays an important role for all forms of cell culture. Sometimes it is the main purpose of the experiment as in toxicity assays, or it can be used to correlate cell behavior to the number of cells (Stoddart, 2011). Cell viability assays are essentially used for screening the response of the cells against a drug or a chemical agent. In particular, pharmaceutical industry widely uses viability assays to evaluate the influence of developed agents on the cells. Researchers apply various types of assays in order to screen the outcome of a developed therapeutics that often target cancer cells (Adan, Kiraz, & Baran, 2016).

There are several types of assays that can be used to determine the number of viable cells. These assays are based on various functions of cells including enzyme activity, cell membrane permeability, cell adherence, adenosine triphosphate (ATP) production, co-enzyme production, and nucleotide uptake activity (Thangaraj, 2016). Although there are different classifications, cell viability assays may be broadly categorized as (a) dye exclusion assays, (b) colorimetric assays, (c) fluorometric assays, (d) luminometric assays, and (e) flow cytometric assays. Dye exclusion assays are the simplest methods that are based on utilization of different dyes such as trypan blue, eosin, congo red, and erythrosine B, which are excluded by the living cells, but not by dead cells. For these assays, although staining procedure is quite straightforward, experimental procedure may be time-consuming in case of large sample sizes. Colorimetric assays are based on the measurement of a biochemical marker to determine the metabolic activity of the cells. In these assays, the colorimetric measurement of cell viability is carried out spectrophotometrically. 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt (WST-1), 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8), lactate dehydrogenase (LDH), sulforhodamine B (SRB), neutral red uptake (NRU), and crystal violet stain (CVS) assays are among the most widely applied colorimetric assays. These assays are simple and economical, and can be applied to both cell suspensions and adherent cells. Fluorometric assays including resazurin and 5-carboxyfluorescein diacetate acetoxymethyl ester (5-CFDA-AM) assays may be performed with a fluorometer, fluorescence microplate reader, fluorescence microscope, or flow cytometer. These assays are advantageous over dye exclusion and colorimetric assays as they are more sensitive. In luminometric assays, a persistent and stable glow-type signal is produced following the addition of reagent. These methods comprise ATP and real-time viability assays (Aslantürk, 2018). Flow cytometry allows simultaneous measurement of the changes in cell morphology by forward and side light scatter, which makes this technology uniquely suited to measuring the complex progression of cell death (Telford, 2012). Major flow cytometric assays include membrane asymmetry (e.g., annexin V and F2N12S staining assays), membrane permeability (e.g., nucleic acid and inclusion and exclusion dyes), and mitochondria assays.

When selecting the appropriate cell viability assay, factors including cost, speed, sensitivity, and the required equipment should be considered in order to obtain reliable results (Shokrzadeh & Modanloo, 2017). An ideal cell viability assay should be safe, rapid, reliable, efficient, and time- and cost-effective, and should not interfere with the test compound (Aslantürk, 2018). On the other hand, regardless of the assay chosen, the most critical factors for accurate and reproducible measurements include (a) the use of a controlled and consistent source of cells to set up experiments and (b) performing suitable characterization of reagent concentration and incubation time for each experimental model system (Riss et al., 2016). Considering the above, in this guideline, the mechanisms and the practice of assessment of the most common cell viability assays applied in research labs are discussed in detail.

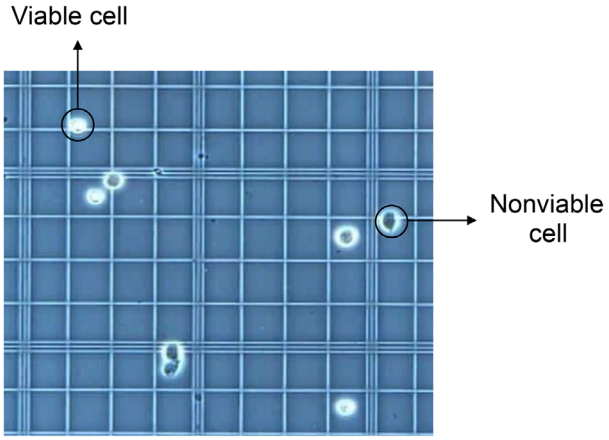

FIGURE 1 Determination of cell viability with trypan blue assay (modified from Allevi Protocols, 2020)

2 | DYE EXCLUSION ASSAYS

2.1 | Trypan blue stain assay

Trypan blue stain assay has initially been developed in 1975 to measure viable cell count and is still used as a confirmatory test for measuring changes in viable cell number caused by a drug or toxin (Tolnai, 1975). Trypan blue stain, a large negatively charged molecule, is one of the simplest assays that are used to determine the number of viable cells in a cell suspension (Stone, Johnston, & Schins, 2009). The principle of this assay is that living cells have intact cell membranes that exclude the trypan blue stain, whereas dead cells do not. Cell suspension is mixed with the trypan blue stain and examined visually under light microscopy to determine whether cells include or exclude the stain. A viable cell will have a clear cytoplasm, whereas a nonviable cell will have a blue cytoplasm (Strober, 2015) (Figure 1).

2.1.1 | Reagent preparation

To perform the trypan blue stain assay, 0.4% trypan blue stain and phosphate-buffered saline (PBS) or serum-free medium are obtained. Trypan blue stain should be stored in dark and filtered after prolonged storage. As trypan blue stain binds to serum proteins and causing misleading results, serum-free medium should be used to obtain reliable results.

2.1.2 | Protocol

The cell suspension to be tested is centrifuged at 100 × *g* for 5 min. The supernatant is discarded and the pellet is resuspended in 1-ml

PBS solution or serum-free medium. Then, one portion of this cell suspension is mixed with one portion of trypan blue stain. The mixture is allowed to stay at room temperature for 3 min. It is important to note that the cells should be counted within 3–5 min of mixing with trypan blue, as longer incubation periods will lead to cell death and hence reduced viability counts. Following the incubation, a drop of the mixture is applied to a hemocytometer, which is placed on the stage of a binocular microscope. Viable, that is, unstained, and nonviable, that is, stained, cells in the hemocytometer are counted separately.

2.1.3 | Calculation

After counting viable and nonviable cells, the total number of viable cells per milliliter of aliquot is determined by multiplying the total number of viable cells by 2, which is the dilution factor for trypan blue. Similarly, total number of cells per milliliter of aliquot is determined by addition of number of viable and nonviable cells and multiplying it by 2. Then, the percentage of viable cells is calculated using the following equation:

$$\%\ \text{Viable cells} = \frac{\text{Total number of viable cells per milliliter of aliquot}}{\text{Total number of cells per milliliter of aliquot}} \times 100.$$

2.2 | Eosin, congo red and erythrosine B stain assays

Eosin is a fluorescent red dye that is used to stain cytoplasm, collagen, and muscle fiber, facilitating their visualization under a microscope (Nakayama & Tsujinaka, 2014). Similarly, congo red is a sulfonated azo dye that is used in microscopy to stain cytoplasm. Erythrosine B, also known as FD&C Red No. 3, is a tetraiodofluorescein dye, which is widely utilized as biological stain and color additive in food and drugs (Kuo et al., 2017). The principle of eosin, congo red, and erythrosine B stain assays also relies on the integrity of the cell membrane as in trypan blue stain assay. Particularly, erythrosine B stain has several advantages over trypan blue stain including (a) being nontoxic, (b) not binding to serum proteins, and (c) not requiring an incubation period prior to counting (Kim et al., 2016).

2.2.1 | Reagent preparation

To perform these assays, eosin, congo red, or erythrosine B stain (0.1%) and PBS are obtained from the manufacturer.

2.2.2 | Protocol

The cell suspension in PBS and the stain are mixed at 1:1 ratio and then the mixture is loaded into a hemocytometer. Nonviable and viable cells, stained red and unstained, respectively, are counted under light microscope.

2.2.3 | Calculation

The calculations are carried out as in trypan blue stain assay using the following equation:

$$\%\ \text{Viable cells} = \frac{\text{Total number of viable cells per milliliter of aliquot}}{\text{Total number of cells per milliliter of aliquot}} \times 100.$$

3 | COLORIMETRIC ASSAYS

3.1 | MTT assay

MTT assay is a simple colorimetric test of cell proliferation and survival, which was developed by Mosmann (1983) and adapted by Cole (1986) for measuring chemosensitivity of human lung cancer cell lines. The assay is based on the conversion of MTT into formazan crystals by living cells, which shows mitochondrial function (Van Meerloo, Kaspers, & Cloos, 2011). It is well known as the first homogeneous cell viability assay was designed for 96-well plates for high-throughput screening. Since then, MTT tetrazolium assay technology has been widely adopted and remains popular in academic labs as evidenced by thousands of published articles (Abbott, 2003). In MTT assay, the tetrazolium salt is reduced to insoluble formazan dye by dehydrogenase enzyme present in the viable cells at 37°C (Figure 2). Further, the insoluble formazan salt is dissolved by the addition of solubilizing agents, and the colored product is quantitatively measured at 570 nm using a spectroscopic multiplate reader. A variety of methods have been used to solubilize the formazan product, stabilize the color, avoid evaporation, and reduce interference by phenol red and other culture medium components (Hall et al., 2004; Wilson & Hay, 2011). Various solubilization methods include the use of acidified isopropanol, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), sodium dodecyl sulfate (SDS), and combinations of detergent and organic solvent (Abbott, 2003; Hall et al., 2004; Wilson & Hay, 2011). The dead cells lose the ability to reduce tetrazolium salts into colored formazan products. Viable cells with active metabolism convert MTT into a purple-colored formazan product with an absorbance maximum near 570 nm. Thus, the intensity of the colored product is directly proportional to the number of viable cells present in the culture (Präbst, Engelhardt, Ringgeler, & Hübner, 2017).

3.1.1 | Reagent preparation

MTT solution is prepared by dissolving MTT in Dulbecco's phosphate-buffered saline (DPBS) at pH 7.4 (5 mg/ml). This solution is filtered and sterilized through a 0.2-μm filter into a sterile and light-protected

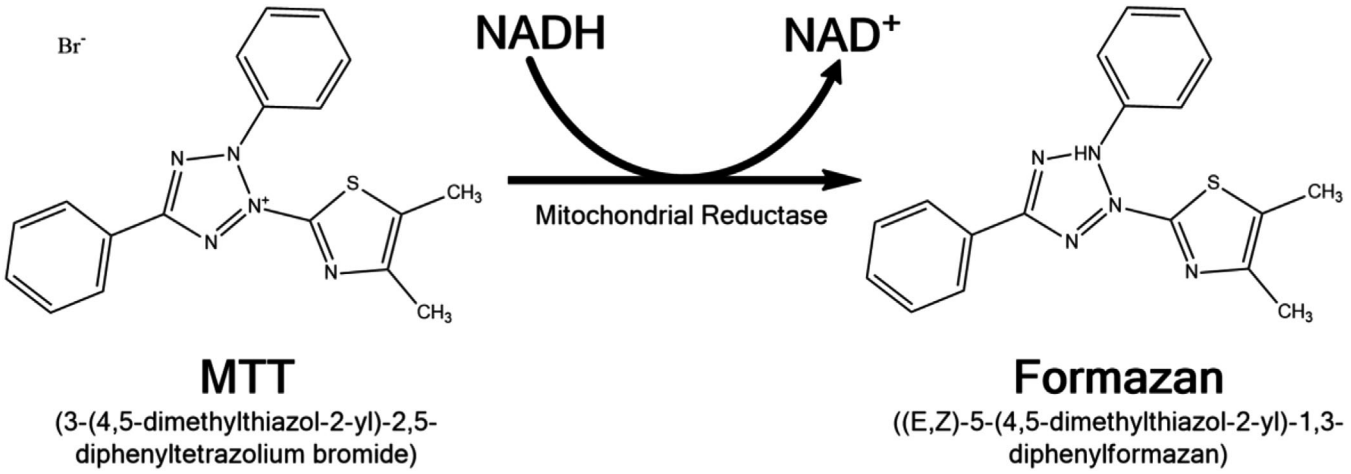

FIGURE 2 Reduction of MTT to formazan crystals

container. MTT solution should be stored at −20°C until analysis or at 4°C for immediate use and should be protected from the light. Solubilization solution is prepared with 40% (v/v) DMF containing 2% (v/v) glacial acetic acid under ventilated fume hood. SDS (16% [w/v]) is added to this solution and pH is adjusted to 4.7. Solubilization solution should be stored at room temperature in order to prevent precipitation of SDS and in case of precipitation it can be heated to 37°C for resolubilization.

3.1.2 | Protocol

Cell suspensions seeded to 96-well plates (100 µl/well) with or without the test compounds are incubated at 37°C in a humidified incubator with 5% CO_2 for required exposure time. MTT solution of 10 µl is added to each well to reach a final concentration of 0.45 mg/ml and incubated at 37°C for 1–4 hr. After incubation, the formazan crystals are dissolved in 100 µl of solubilization solution and the absorbance is measured at 570 nm with a multiplate reader.

3.1.3 | Calculation

The percentage of cell viability is calculated using the following equation:

$$\%\,\text{Viability} = \frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{blank}}} \times 100.$$

3.2 | MTS assay

MTS has initially been developed as a new tetrazolium analog of MTT as a substitute for MTT in the microculture screening assay for in vitro cell growth (Cory, Owen, Barltrop, & Cory, 1991). It is categorized in a more recently developed group of tetrazolium reagents that can be reduced by viable cells to produce formazan products directly soluble in the cell culture medium (Barltrop, Owen, Cory, & Cory, 1991; Goodwin, Holt, Downes, & Marshall, 1995). Such enhanced tetrazolium reagent removes liquid handling during the assay process because a second application of the reagent to the assay plate is not required to solubilize the precipitate of formazan, rendering the protocols easier. This group of tetrazolium reagents is also used in combined application with intermediate electron-acceptor reagents such as PMS (phenazine methyl sulfate) or PES (phenazine ethyl sulfate) that can penetrate viable cells, lessen cytoplasm, or cell surface, and exit the cells in which tetrazolium can be converted to a dissolved formazan product (Berridge, Herst, & Tan, 2005).

3.2.1 | Reagent preparation

MTS solution is prepared by dissolving 2 mg/ml of MTS powder in DPBS until a clear yellow solution is obtained. PES powder is dissolved in MTS solution to 0.21 mg/ml, and the pH is adjusted to 6.0–6.5 with 1 N HCl. This solution is filtered and sterilized through a 0.2 µm filter into a sterile and light-protected container. MTS solution should be stored at −20°C until analysis or at 4°C for immediate use and should be protected from light.

3.2.2 | Protocol

Cell suspensions seeded to 96-well plates (100 µl/well) with or without the test compounds are incubated at 37°C in a humidified incubator with 5% CO_2 for required exposure time. MTS solution of 20 µl is added to each well to reach a final concentration of 0.33 mg/ml and incubated at 37°C for 1–4 hr. After incubation, the absorbance is measured at 490 nm with a multiplate reader.

3.2.3 | Calculation

The percentage of cell viability is calculated using the following equation:

$$\% \text{ Viability} = \frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{blank}}} \times 100.$$

3.3 | XTT assay

XTT has been synthesized by Paull et al. (1988). Bioreduction of XTT yields a highly colored formazan product with only viable cells. In contrast to other tetrazolium salts such as MTT, formazan dye is soluble in aqueous solutions and directly quantified using a scanning multiplate spectrophotometer (ELISA reader). This enables a high degree of accuracy, allows online data processing by computers (data collection, calculation, and reporting generation), and thus allows a high number of samples to be handled quickly and conveniently. Cells are incubated with the yellow XTT solution in a 96-well tissue culture plate. During this time of incubation, orange formazan solution is produced and is quantified spectrophotometrically utilizing an ELISA plate test. An increase in the number of living cells results in an increase of the sample's total activity of mitochondrial dehydrogenases. This rise is closely associated with the quantity of formed orange formazan, as measured by the absorbance (Paull et al., 1988; Scudiero et al., 1988).

3.3.1 | Reagent preparation

XTT working solution was prepared, immediately before use, by dissolving l mg/ml XTT in sterile Hanks' balanced salt solution followed by addition of PMS at 5 µL/ml (5 mM stock solution).

3.3.2 | Protocol

Cell suspensions seeded to 96-well plates (10,000 cells/well) with or without the test compounds (200 µl/well) are incubated at 37°C in a humidified incubator with 5% CO_2 for required exposure time. At the end of incubation, 100 µl of XTT solution mix was added to each well (final concentration = 0.3 mg/ml), and plates were incubated at 37°C for 4 hr. Absorbance was measured at 450 nm against a reference wavelength at 650 nm using a microplate reader.

3.3.3 | Calculation

The percentage of cell viability is calculated using the following equation:

$$\% \text{ Viability} = \frac{(A_{450} - A_{650}) \text{ of test cells}}{(A_{450} - A_{650}) \text{ of control cells}} \times 100.$$

3.4 | WST-1 assay

WST-1 has been developed by Ishiyama, Shiga, Sasamoto, Mizoguchi, and He (1993). It is a tetrazolium salt that produces a highly water-soluble formazan by mitochondrial dehydrogenase enzymes in the presence of intermediate electron acceptor, such as 1-methoxy PMS. The amount of formazan produced is directly proportional to the amount of mitochondrial dehydrogenase in cell culture. Thus, the assay measures the metabolic activity of cells. WST-1 has a similar sensitivity to XTT, whereas it is less toxic compared to XTT. Furthermore, WST-1 does not require an additional step to dissolve the formazan as in case of MTT, which is advantageous when large-scale drug screening is attempted (Ishiyama et al., 1993).

3.4.1 | Reagent preparation

WST-1 reagent solution is prepared as an aqueous solution containing 5 mM WST-1, 0.2 mM 1-methoxy PMS, and 12.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.0).

3.4.2 | Protocol

Cell suspensions seeded to 96-well plates (100 µl/well) with or without the test compounds are incubated at 37°C in a humidified incubator with 5% CO_2 for required exposure time. Then, 10 µl of WST-1 reagent solution is added to each well and the plate is incubated at 37°C for 2 hr. After incubation, the absorbance is measured at 450 nm with a multiplate reader.

3.4.3 | Calculation

The percentage of cell viability is calculated using the following equation:

$$\% \text{ Viability} = \frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{blank}}} \times 100.$$

3.5 | WST-8 assay

WST-8 is a second-generation tetrazolium salt that was first synthesized by Tominaga et al. (1999). It is used as a chromogenic indicator for cell viability. The reduction of slightly yellow WST-8 by viable cells produces an orange-colored formazan product, which is directly proportional to the number of viable cells in the range of 200–25,000 cells/well for many cell lines including nonadherent cells. WST-8 is found to be more sensitive for cell viability measurements than those of other tetrazolium salts including MTT, MTS, XTT, and WST-1. Furthermore, WST-8 produces water-soluble formazan upon cellular reduction, which does not require an additional step to dissolve the

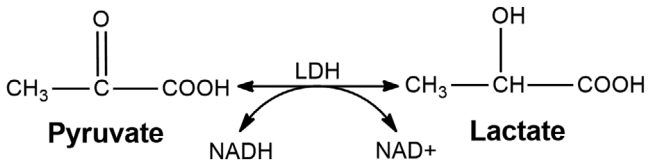

FIGURE 3 Conversion of lactate to pyruvate catalyzed by lactate dehydrogenase (LDH)

formazan, providing an additional advantage to the method (Tominaga et al., 1999).

3.5.1 | Reagent preparation

WST-8 reagent solution is prepared as an aqueous solution containing 5 mM WST-8, 0.2 mM 1-methoxy PMS, and 150 mM NaCl.

3.5.2 | Protocol

Cell suspensions seeded to 96-well plates (100 µl/well) with or without the test compounds are incubated at 37°C in a humidified incubator with 5% CO_2 for required exposure time. Then, 10 µL of WST-8 reagent solution is added to each well and the plate is incubated at 37°C for 2 hr. After incubation, the absorbance is measured at 450 nm with a multiplate reader.

3.5.3 | Calculation

The percentage of cell viability is calculated using the following equation:

$$\%\ \text{Viability} = \frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{blank}}} \times 100.$$

3.6 | LDH assay

LDH assay was developed in the 1980s as a rapid and sensitive method for assaying cytotoxicity in immune cells (Decker & Lohmann-Matthes, 1988). LDH is a stable cytoplasmic enzyme that is released into the cell culture medium due to the loss of membrane integrity (Chan, Moriwaki, & De Rosa, 2013), which is a typical characteristic of cells undergoing apoptosis, necrosis, or other forms of cellular damage. LDH oxidizes reduced form of nicotinamide adenine dinucleotide (NADH), generating NAD+, and catalyzes the conversion of lactate to pyruvate (Figure 3). In this protocol, NADH reduces the yellow tetrazolium salt, INT (iodonitrotetrazolium or 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium), into a water-soluble red formazan dye. The amount of formazan is determined considering the absorbance values measured at 490 nm, which represents the total LDH activity in the culture and it is directly proportional to the number of damaged cells (Kumar, Nagarajan, & Uchil, 2018).

3.6.1 | Reagent preparation

To preform LDH assay, substrate, lysis, and stop solutions are prepared. For the preparation of substrate solution, first INT and 1-methoxy PMS (1-methoxyphenazine methosulfate) are dissolved in PBS to a final concentration of 100 mM. Then, 0.054 M L-(+)-lactic acid, 1.3 mM β-NAD+, 0.66 mM INT, and 0.28 M 1-methoxy PMS solutions dissolved in 0.2 M Tris–HCl buffer at pH 8.2 are combined to obtain the substrate solution. It is important to note that the substrate solution should be prepared as fresh prior to each experiment. The lysis solution is prepared with 9% (v/v) Triton X-100, whereas to prepare the stop solution, 50% DMF and 20% SDS at pH 4.7 are used. Alternatively, 1 N HCl can also be used to stop the reaction; however, DMF–SDS solution is advantageous in cell culture medium containing Phenol Red, as it neutralizes the background absorption. Interference of background absorption can also be eliminated using a Phenol Red- free medium.

3.6.2 | Protocol

As different cell lines comprise diverse amounts of LDH, a preliminary study should be performed to ensure the optimum number of cells. Accordingly, various cell dilutions (0–20,000 cells/well) are prepared and 100 µl of the cell suspensions are added to 96-well plate. Afterward, 15 µl of lysis solution is added to each well and the plate is centrifuged at 250 × *g* for 4 min. Then, 50 µl of the supernatants are transferred to 96-well flat-bottom enzymatic assay plate followed by the addition of 50 µl substrate solution. The plate is covered to protect it against the light and incubated at 37°C for 15–30 min. After incubation, 100 µl of stop solution is added and within 1 hr of stop solution addition, the absorbance is measured at 490 nm using a plate reader. The background absorbance is set at 690 nm and subtracted from the measurements carried out at 490 nm. At this point, it is important to ensure that no bubbles are formed in the wells. The cell concentration with absorbance values at least two times the background absorbance of the control medium is determined as the optimum cell number. The assay should be performed at least in triplicates.

3.6.3 | Calculation

The absorbance values obtained for the optimum cell concentration is used for the determination of the percent of cell death (% cytotoxicity) using the following equation:

$$\%\ \text{Cytotoxicity} = \frac{\text{Experimental LDH release } (OD_{490})}{\text{Maximum LDH release } (OD_{490})} \times 100.$$

Alternatively, LDH standard can be used to express the results. Accordingly, 50 µl of assay substrate is added to 50 µl of different dilutions of LDH standard dissolved in cell culture medium (0.2–2.0 U/ml). The mixture is incubated for 15 min and the absorbance values are measured as indicated above. A standard curve is plotted using the obtained data and it is used to calculate the enzyme activity of the tested samples.

3.7 | SRB assay

SRB assay has initially been developed in 1990 to evaluate the cytotoxicity of anticancer drugs (Skehan et al., 1990). SRB is a bright-pink aminoxanthene dye with two sulfonic groups that bind to amino-acid residues under mild acidic conditions, and dissociate under basic conditions. This protocol is based on the binding ability of SRB to cellular proteins, which is fixed using TCA (trichloroacetic acid). The protein-bound dye is then dissolved in Tris-base (tris(hydroxymethyl)aminomethane) solution and the absorbance values measured at 510 nm are used to determine the number of viable cells (Vichai & Kirtikara, 2006).

3.7.1 | Reagent preparation

To preform SRB assay, 10% (w/v) TCA solution, 0.057% (w/v) SRB dissolved in 1% (v/v) acetic acid, 1% (v/v) acetic acid solution, and 10 mM unbuffered Tris-base solution at pH 10.5 are prepared for fixation, staining, washing, and dissolution steps, respectively.

3.7.2 | Protocol

Cell suspensions seeded to 96-well plates (19,000 cells/well) with or without the test compound (10 µl dissolved in 10% DMSO) are incubated at 37°C in a humidified incubator with 5% CO_2 for 72 hr. The cells attached to the bottom of the wells are fixed with addition of 100 µl of cold TCA and subsequent incubation at 4°C for 1 hr. After 1 hr, the plate is washed four times with slow-running tap water. The excess water is removed using paper towel and a blow dryer is used to dry the plate completely. Afterward, 100 µl of the SRB solution is added to the cells and the plate is incubated at room temperature for 30 min. After 30 min of incubation, the unbound SRB stain is removed by washing the wells four times with acetic acid solution. Again, a blow dryer is used to completely dry the plate and then SRB stained cells are dissolved in 200 µl of unbuffered Tris-base solution. The plate is shaken for 5 min to solubilize the protein-bound SRB dye. Another option is to incubate the plate for 30 min in Tris-base solution for complete solubilization of SRB dye. Then, the absorbance values are recorded at 510 nm using a plate reader. Alternatively, the measurement can be done fluorometrically at excitation and emission wavelengths of 488 and 585 nm, respectively. The assay should be performed at least in triplicates.

3.7.3 | Calculation

The percentage of cell-growth and growth inhibition are calculated using the following equations:

$$\%\ \text{Cell growth} = \frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{blank}}} \times 100,$$

$$\%\ \text{Growth inhibition} = 100 - \%\ \text{Cell growth}.$$

3.8 | NRU assay

NRU assay has been developed to quantify viable cells in monolayer cultures (Borenfreund & Puerner, 1985). This protocol is based on the binding ability of viable cells to the supravital dye neutral red (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride) in the lysosomes. The bound dye is then extracted from the viable cells using an acidified ethanol solution, and the absorbance of the solubilized dye is measured using a spectrophotometer (Repetto, Del Peso, & Zurita, 2008).

3.8.1 | Reagent preparation

To preform NRU assay, neutral red working solution dissolved in PBS (40 µg/ml) and destain solutions are prepared. Neutral red destain solution contained 50% ethanol (96%), 49% deionized water, and 1% glacial acetic acid (v/v). In addition, optionally, 5% glutaraldehyde may be prepared by dilution of the commercial 25% for fixation purpose.

3.8.2 | Protocol

Cell suspensions seeded to 96-well plates (approximately 50,000 cells/well) are incubated overnight to form a half-confluent monolayer. Next day, the medium is removed from the plate and the cells are treated or untreated with the test compounds, followed by another incubation for overnight. At this stage, neutral red working solution is also prepared and incubated overnight at the same temperature as the cells. Following incubation, the neutral red solution is centrifuged at 600 × *g* for 10 min to remove any precipitated dye crystals. The medium is removed from the cells and 100 µL of neutral red solution is added to each well. The plate is incubated for 2 hr under proper culture conditions and then the red medium is removed and the cells are washed with 150 µl PBS. For low-adherent cell cultures, it is recommended to also include a fixation step with 5% glutaraldehyde for 2 min prior to washing. Afterward, 150 µl of neutral red detain solution is added to each well and the plate is shaken for 10 min to extract the neutral red dye from the cells and to obtain a homogeneous solution. Then, the absorbance values are recorded at 540 nm with a plate reader spectrophotometer using blanks that contain no cells as a reference. Alternatively, the measurement can be done fluorometrically at excitation and emission wavelengths of 530 and 645 nm, respectively. The assay should be performed at least in triplicates.

3.8.3 | Calculation

The percentage of cell viability is calculated using the following equation:

$$\%\ \text{Viability} = \frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{blank}}} \times 100.$$

3.9 | CVS assay

CVS assay was developed by Saotome, Morita, and Umeda (1989) for evaluating the cytotoxicity of chemicals. It is used for the indirect quantification of cell death. The protocol is based on the determination of the adherence of cells through staining of attached cells with CVS, which binds to proteins and DNA. The dead cells lose their ability to adhere, which results in the reduction of the amount of CVS in the cell culture. The bound dye is extracted from the viable cells using methanol, and the absorbance of the solubilized dye is measured using a spectrophotometer (Feoktistova, Geserick, & Leverkus, 2016).

3.9.1 | Reagent preparation

To preform CVS assay, 0.5% CVS solution is prepared by dissolving crystal violet powder in 20% aqueous methanol. CVS solution can be stored at room temperature up to 2 months.

3.9.2 | Protocol

Cell suspensions seeded to 96-well plates (10,000–20,000 cells/well) are incubated at 37°C for 18–24 hr to enable adhesion of cells to wells. Wells without cells are also prepared to serve as a control to avoid nonspecific binding of the CVS. After incubation, the medium is removed from the plate and 100 µl of test compound at varying concentrations are added to the wells, followed by another incubation at 37°C for 18–24 hr. Afterward, the medium is removed and the plate is washed two times with slow-running tap water. The excess water is removed gently by inverting the plate on a filter paper. Then, 50 µl of CVS solution is added to each well and the plate is incubated at room temperature for 20 min on a bench rocker with a frequency of 20 oscillations per minute. After 20 min of incubation, the unbound CVS is removed by washing the wells four times with tap water. Again, the plate is inverted on a filter paper to remove excess water and then air-dried for minimum of 2 hr. Once the plate becomes completely dry, 200 µl of methanol is added to each well and incubated at room temperature for 20 min on a bench rocker with a frequency of 20 oscillations per minute. Then, the absorbance values are recorded at 570 nm using a plate reader. The assay should be performed at least in triplicates.

3.9.3 | Calculation

The average absorbance values of wells without cells are subtracted from the absorbance values of wells with cells. Then, the percentage of cell viability is calculated using the following equation:

$$\% \text{ Viability} = \frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{blank}}} \times 100.$$

4 | FLUOROMETRIC ASSAYS

Fluorometric assays are developed in 1990s as an alternative to exclusion dyes and colorimetric methods. Fluorometric cell viability methods are based on the nonspecific cleavage of a nonfluorescent compound such as fluorescein diacetate which fluoresces following its cleavage by cellular esterases. Nascent fluorescent signal is then measured to determine the amount or the ratio of the viable cells. Fluorometric assays are easy to perform and relatively cheap but fluorescent interference caused by the applied test compounds is possible (Altman, Randers, & Rao, 1993; Rotman & Papermaster, 1966).

4.1 | Resazurin (alamar blue) assay

The resazurin-based test was first used to examine the sanitary state of milk in the late 1920s (Ali-Vehmas, Louhi, & Sandholm, 1991; Nixon & Lamb, 1945; Twigg, 1945). After that, it has been used for plant metabolism studies (De Jong & Woodlief, 1977), semen quality evaluations (Glass et al., 1991), and antifungal susceptibility tests (To, Fothergill, & Rinaldi, 1995). It has also been a valuable method for analyzing toxicants owing to several advantages of the assay (Hamid, Rotshteyn, Rabadi, Parikh, & Bullock, 2004; O'Brien, Wilson, Orton, & Pognan, 2000; Perrot, Dutertre-Catella, Martin, Rat, & Warnet, 2003). Moreover, the safety, simplicity, homogeneity, and sensitivity of this method offer predominance over the other classic tests used to measure cell viability and proliferation (Jonsson, Frost, Larsson, Ljunghall, & Ljunggren, 1997; Larson, Doughman, Gregerson, & Obritsch, 1997).

Alamar blue fluorometric assay is based on the nonspecific, enzymatic, irreversible reduction of the compound by viable cells. Following the enzymatic reaction within the cells, alamar blue or resazurin is reduced into resorufin, which is pink and extracted from the living cells into the medium. The extracted compound results in a change in the color of the medium and color change can be measured from 50 up to 50,000 cells in a linear range, using 530–570 nm for excitation/580–620 nm for emission fluorescent filters. The alamar blue method is sensitive, simple, and safe to monitor the cell viability and proliferation (Czekanska, 2011; Johnson, Nguyen, & Coder, 2013; Larson et al., 1997). There are several commercially available alamar blue cell viability and proliferation kits such as alamarBlue Cell Viability Reagent from Thermo Fisher Scientific, alamarBlue from Bio-Rad, in vitro Toxicology Assay Kit from Sigma–Aldrich, AlamarBlue Cell Viability Assay Reagent from G-Biosciences, Cell-Quant AlamarBlue Cell Viability Reagent from GeneCopoeia, and so forth.

4.1.1 | Reagent preparation

Alamar blue solution can also be prepared from the powder instead of using commercially available kits. Alamar Blue high-purity powder is dissolved in PBS (pH 7.4) to 0.15 mg/ml. Alamar blue solution can be sterilized via filtering and can be stored at 4°C for short-term storage and at −20°C for long-term storage (Riss et al., 2016).

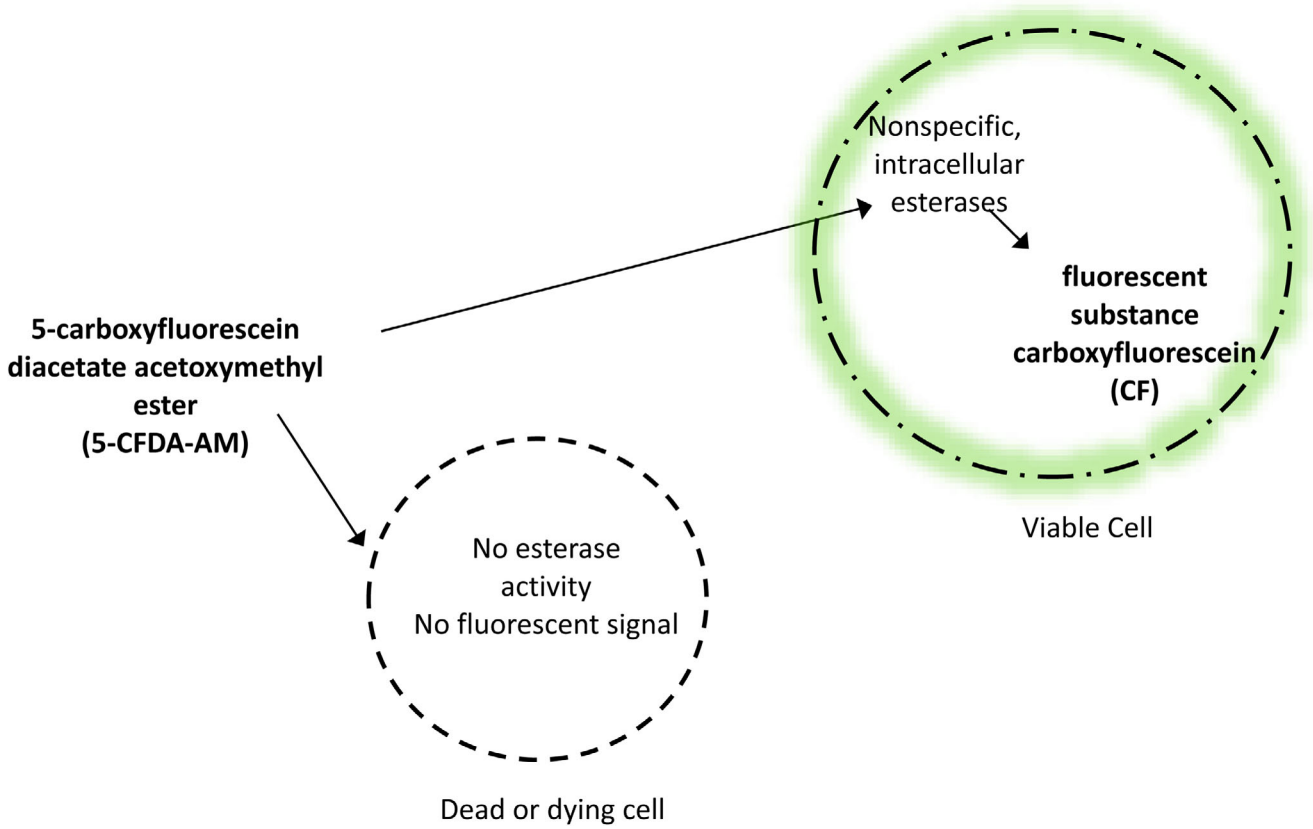

FIGURE 4 Schematic illustration of the principles of 5-carboxyfluorescein diacetate acetoxymethyl ester (5-CFDA-AM) assay. 5-CFDA-AM is a target of intracellular nonspecific esterase enzymes in living cells. Following the nonspecific enzymatic activity of esterases, 5-CFDA-AM is converted into fluorescent substance carboxyfluorescein (CF), which is polar and nonpermeable through the cellular membrane of living cells. Dead or dying cells lack the esterases and membrane permeability control so that they do not have any fluorescent signal

4.1.2 | Protocol

Alamar blue cell viability and proliferation assay can be applied to a number of cell types but it is not recommended to run the assay immediately after thawing the cells from cryopreservation. Cell density optimizations should be assessed before running the assay depending on the cell type (adherent/suspension) and cell size (hepatocytes, stem cells or neuronal cells, etc.).

After splitting the cells into the corresponding wells with the culture medium, cells are incubated at least for 5 hr at 37°C, supplemented with 5% CO_2. Incubation time can be adjusted for treatment conditions and can be longer. Noncell controls are included to check the background fluorescence and nontreated/sham-treated controls constitute the negative controls. Following the incubation, 10% (v/v) alamar blue solution is added into the each well. If the cells are adherent, culture medium can be aspirated and replaced by a new medium mixture containing 10% (v/v) alamar blue. Alamar blue solution should be kept in a light-shield tube and once the solution is added into the wells, culture plates should be covered with aluminum folio. Cells are incubated with alamar blue solution for 4 hr at 37°C, supplemented with 5% CO_2.

After the incubation, 150 µl medium is transferred into a new 96-well plate (duplicates for each sample) and fluorescence is measured at excitation and emission wavelengths of 530–570 and 580–620 nm, respectively, on the plate reader. For calculation, average value of no-cell control is subtracted from each well. A cell number standard curve should be prepared for each cell type for the analysis of obtained values if the exact cell number calculation is necessary (Czekanska, 2011; Riss et al., 2016). In addition to exact cell number calculation, the percentage of cell growth and growth inhibition can also be calculated using the equations described in the previous sections.

4.2 | 5-CFDA-AM assay

5-CFDA-AM is another compound used in fluorometric cell viability assays. Similar to alamar blue, 5-CFDA-AM is a target of intracellular nonspecific esterase enzymes in living cells. Following the nonspecific enzymatic activity of esterases, 5-CFDA-AM is converted into fluorescent substance carboxyfluorescein, which is polar and nonpermeable through the cellular membrane of living cells (Ganassin & Bols, 2000) (Figure 4). Examples of commercially available 5-CFDA-AM probes are as follows: 5-CFDA-AM (5 mg) from Invitrogen, 5-CFDA, AM from Synchem, 5-CFDA (5-Carboxyfluorescein diacetate, single isomer, 100 mg) from Biotium, and so forth.

4.2.1 | Reagent preparation

5-CFDA, AM stock solution of 4 mM can be prepared in anhydrous DMSO. Stock solution can be aliquoted and stored at −20°C but the solution must be protected from light and moisture (Ganassin & Bols, 2000).

4.2.2 | Protocol

Cells should be plated into culture plates that are also compatible with plate readers. Wells without cells should be included to monitor the background fluorescence. Stock solution of 4 mM 5-CFDA, AM is diluted (1:1,000) using the serum- and amino acid-free culture medium.

Culture medium is aspirated from the wells of adherent cells and diluted 5-CFDA, AM solution, 4 μM working solution, is added to the respective cells. For suspension cell cultures, 8 μM 5-CFDA, AM working solution can be prepared via 1:500 dilution of 4 mM stock solution in serum- and amino acid-free culture medium. Cells are split into the wells using serum- and amino acid-free culture medium and then equal amount of 8 μM 5-CFDA, AM working solution is added to the corresponding wells. For example, for 96-well plate cultures, cells are split into wells within 100 μl serum- and amino acid-free culture medium and then 100 μl of 8 μM 5-CFDA, AM working solution is added to each well.

Cells are incubated with 5-CFDA, AM for 30 min at 18–22°C in the dark. Following incubation, fluorescence is measured at excitation and emission wavelengths of 493 and 541 nm, respectively, using a fluorescence plate reader (Ganassin & Bols, 2000; Schirmer, Chan, Greenberg, Dixon, & Bols, 1997; Schreer, Tinson, Sherry, & Schirmer, 2005).

The percentage of cell growth and growth inhibition can be calculated using the equations described in the previous sections. If exact cell number calculation is necessary, a cell number standard curve should be prepared for each cell type for the analysis of obtained values.

5 | LUMINOMETRIC ASSAYS

The bioluminescence assays are based on the correlation between a bioluminescent reaction and the effect of a tested compound. This effect can be an increase in cell proliferation or cell death (Bulich & Isenberg, 1981; Voyevodina, Nifantyev, Kovalevsky, Schultz, & Kratasyuk, 1990). Bioluminescent measurements are performed using luminometers since 1970s. Modern luminometers carry a photon counter and the obtained signal is proportional, but not equal, to the emitted photons (Lundin, 2000).

5.1 | ATP assay

ATP bioluminescence has initially been developed to determine whether there was a linear relationship between cultured cell number and measured luminescence using the luciferin–luciferase reaction (Crouch, Kozlowski, Slater, & Fletcher, 1993). Intracellular ATP is a valid indicator of cell viability. ATP synthesis is interrupted and remaining ATP is depleted by ATPases immediately once the cells loose membrane integrity and cell viability (Ugarova, Brovko, Trdatian, & Raĭnina, 1987). Intracellular ATP concentration may vary with the cellular stress factors, physiological changes such as treatments, and cell viability (Ugarova, 1993). For the detection of ATP concentration, luciferase assay can be applied as ATP is a necessary component for the oxidation reaction of luciferin (McElroy, 1947). ATP-coupled luciferase reaction can be summarized as follows (Lomakina, Modestova, & Ugarova, 2015):

$$\text{D}-\text{Luciferin} + \text{O}_2 + \text{ATP} + \text{Mg}^{+2} \xrightarrow{\text{Firefly luciferase}} \text{oxyluciferin} + \text{AMP} + \text{PPi} + \text{lightquantum}\,(\sim 560\text{nm})\,.$$

In the luminometric ATP cell viability assays, cells first get permeable to ATP so that luciferase enzyme can interact with intracellular ATP. Then intracellular ATPases are inactivated and finally the light is measured via luminometers to determine the intracellular ATP levels (Figure 5). Luminescent signal is quite stable and can be measured within a few hours and most of the assays are very specific that the signal can be measured even from 50 cells. Examples of commercially available luminometric ATP assays are as follows: ATP Assay Kit – Luminometric from Assay Biotech, ATP Determination Kit from ThermoScientific, Luminescent ATP Detection Assay Kit from Abcam, CellTiter-Glo Luminescent Cell Viability Assay from Promega, Rapid Luminometric ATP Assay Kit from AAT Bioquest, and so forth.

5.1.1 | Protocol

Commercially available luminometric ATP cell viability assays are relatively cheap, easy to perform, minimize the technical errors, and give reproducible data, so that the manual preparation of the assay is almost fully replaced by the kits. In general, kits are designed for 96- or 384-well plates.

Cells are split into 96-well plates in 100 μl (25 μl for 384-well plate) culture medium containing desired treatment compounds or conditions. Like previously described cell viability assays, no-cell wells should be included for blank subtraction and no-treatment cells should be included as controls. After desired incubation or treatment period, plates are equilibrated to room temperature for 30 min. Most of the ready-to-use kits have only one working solution containing all compounds of the assay: detergents, ATPase inhibitors, luciferin, and luciferase (Assay Biotech, AAT Bioquest, Promega, and ThermoScientific [Abcam, Cambridge, UK]). After incubation at room temperature, equal volume of assay buffer (100 μl for 96-well plate and 25 μl for 384-well plate) is added to each well and incubated on an orbital shaker at room temperature for 10–20 min. If the kit has separate solutions (like Luminescent ATP Detection Assay Kit from Abcam), 50 μl

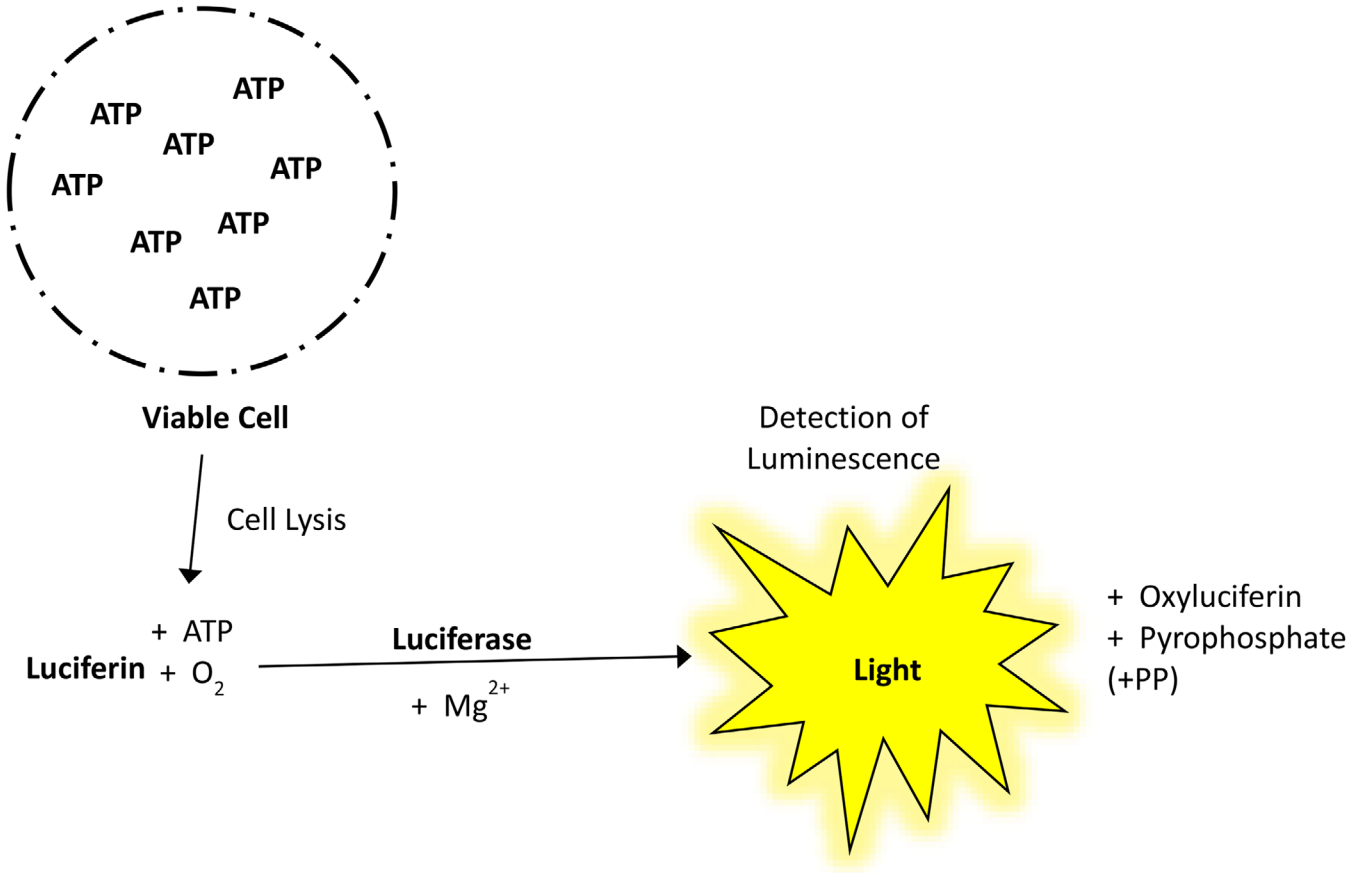

FIGURE 5 Schematic illustration of the principles of ATP assay. ATP is an essential component of living cells. ATP synthesis is interrupted and remaining ATP is depleted by ATPases immediately once the cells loose membrane integrity and cell viability. In the luminometric ATP cell viability assays, cells first get permeable to ATP so that luciferase enzyme can interact with intracellular ATP. ATP is a necessary component for the oxidation reaction of luciferin and coupled reaction results in oxyluciferin, pyrophosphate, and light. The light is measured via luminometers to determine the intracellular ATP levels

detergent–ATPase inhibitor buffer is added first and after incubation 50 µl luciferin–luciferase buffer is added and incubated (96-well plate).

For calculation, ATP standard curve is prepared using the standard ATP stock solution. A standard curve with 10 pM to 10 µM range is anticipated to be sufficient for comparison. Working solution is also added to the standard curve wells and the standard curve plate is incubated at room temperature for the same period as the experimental groups.

Following incubation, luminescence is measured by a luminometer at 560 nm wavelength.

5.2 | Real-time viability assay

Real-time viability assay is a new approach of luciferase method and is the only cell viability method that allows to monitor the cell viability in real time. In this new approach, a marine shrimp-derived engineered luciferase and a pro-substrate of luciferase are used. In this method, cell-permeable pro-substrate and luciferase are added into the culture medium as well but cells are not lysed to release the intracellular ATP. Instead, viable cells uptake pro-substrate and convert it into "substrate" that diffuses into the culture medium. Then, luciferase enzyme uses diffused substrate and generates luminescent signal. This method can be applied for both continuous measurement applications and end-point assays (Aslantürk & Çelik, 2017; Riss et al., 2016).

Examples of commercial real-time viability assay kits are RealTime-Glo MT Cell Viability Assay from Promega and MSCGlo Real Time from HemoGenix.

5.2.1 | Protocol

Cells are split into the wells with medium containing desired treatment compound and real-time viability assay buffer. Cells are then incubated at 37°C and continuous luminescence measurements are recorded every 30 min or every hour depending on the experimental design. Real-time cell viability assay buffers are stable at 37°C up to 72 hr but an optimization step is necessary for each cell type to determine the time period in which limiting factor pro-substrate is consumed totally. For end-point experiments, real-time viability buffer

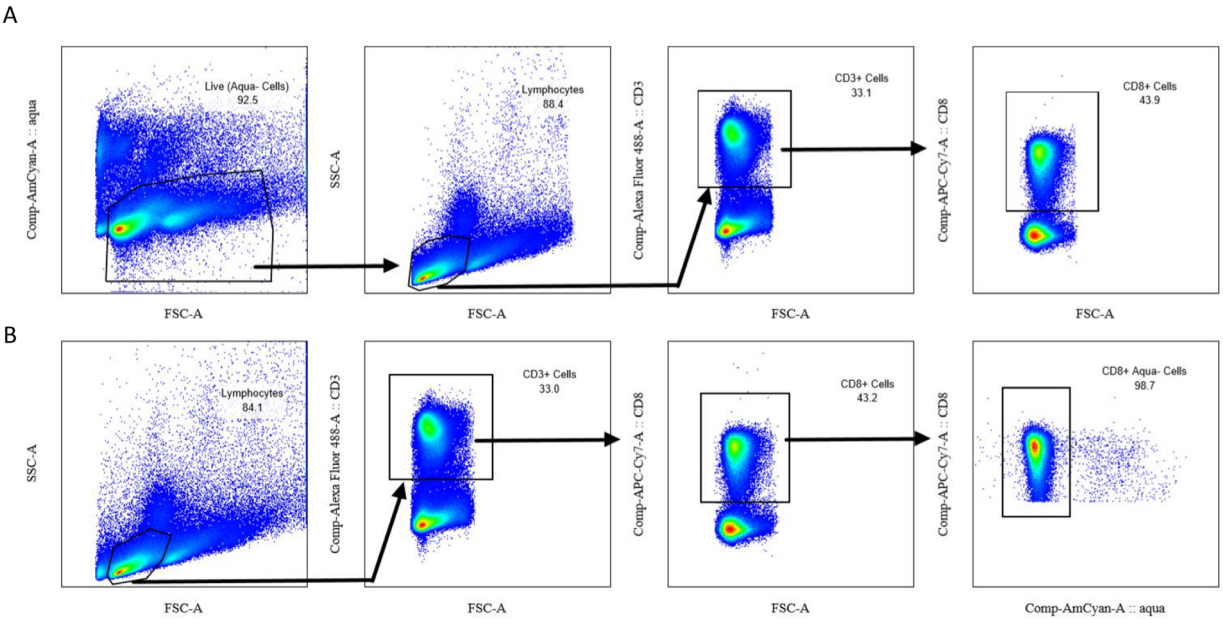

FIGURE 6 Surface staining of mouse splenocytes combined with LIVE/DEAD Fixable Aqua Dead Cell Stain (ThermoFisher Scientific). A, Gating strategy may start with eliminating the dead cells first and continue with FSC–SSC and phenotyping markers CD3 and CD8. B, Gating can also start with selecting populations with FSC–SSC and phenotyping markers CD3 and CD8. After selecting $CD8^+$ cells, the viable population is selected via eliminating Aqua positive cells. Splenocytes were analyzed with FACS Canto and FlowJo Software (10.6.2. for Windows 10, ©Becton, Dickinson and Company [BD]) was used to analyze flow cytometry data. FITC antimouse-CD3 clone 17A2 and APC/Cyanine7 antimouse CD8a clone 53-6.7 were both from Biolegend (unpublished data)

is added into the wells at the end of the treatment conditions. Cells are incubated with the buffer at 37°C for 10 min to 1 hr and then luminescent signal is measure (Riss et al., 2016). For cell viability calculation, standard ATP measurement or percentage cell growth can be used.

6 | FLOW CYTOMETRIC ASSAYS

Development of first flow cytometry machines dates back to 1950s. First flow cytometry was designed as simple cell counter but two-parameter flow cytometry applications were developed immediately. The principle of flow cytometry is characterizing or phenotyping the cells within a liquid flow through lasers. In other words, flow cytometry is a quantitative single cell analysis. Cells can be characterized depending on the size, granularity, and whether carrying a specific fluorescent molecule or not (Macey, 1988). Flow cytometry method has been extensively applied to cell viability and toxicity studies. First of all, changes in forward versus side scatter (FSc vs. SSc) can be analyzed without using any fluorescent dyes; dying cells are usually smaller than viable cells and they present increased SSc. Fluorescent staining targeting different properties of living and healthy cells is performed for more specific phenotypic cell viability analysis. In flow cytometry, cell viability and toxicity analysis can be easily combined with other phenotypic stainings to determine the differentiation status of a cell type, to analyze a specific cell type from a mixed culture, to determine the immune activation status, and so forth (Figure 6).

6.1 | Membrane asymmetry assays

In the early stages of the cell death, especially with apoptosis, cells preserve the membrane permeability and integrity, but the composition of the outer surface of cell membrane is altered: phospholipids become loosely packed and phosphatidylserine starts to appear at the outer surface of the cell membrane. Outer surface phosphatidylserines can be detected by fluorescently labeled annexin V, which is a Ca^{2+}-dependent phospholipid-binding protein (Van Engeland, Ramaekers, Schutte, & Reutelingsperger, 1996).

A more sophisticated solution for the detection of the changes on cellular membrane can be obtained via wavelength ratiometry (Demchenko, 2010). F2N12S (4′-*N*,*N*-diethylamino-6-(*N*-dodecyl-*N*-methyl-*N*-(3-sulfopropyl))ammoniomethyl-3-hydroxyflavone) probe is a small molecule that has dual-color fluorescence emission. As a result of its dual-color fluorescence emission, F2N12S is a self-referencing probe; obtained ratio is independent of cell size or probe concentration. F2N12S probe is incorporated into the cell membrane phospholipids and produce green emission (at 515–545 nm). Once the cells become apoptotic, cellular membrane charges are altered and green emission shifts toward orange emission (at 564–606 nm). The ratio of green to orange emission is used to measure viability percentage or ratio. As F2N12S probe is a small molecule, it is not sensitive to proteases. The staining takes only 5 min and F2N12S binding is Ca^{2+} independent (Demchenko, 2013).

Both annexin V and F2N12S probe target cell surface so that membrane permeabilization is not required and both stainings can be

combined with other markers either for cell surface phenotyping or for intracellular functional analysis.

Both annexin V kits can be found from different distributers. A few examples of annexin V kits are as follows: Annexin V Staining Kits from Abcam (APC, FITC), TACS Annexin V-FITC Apoptosis Detection Kit from Biotechne, Annexin V kit from Bio-Rad (FITC, Biotin, PE, APC), Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit from ThermoFisher Scientific, Annexin V-FITC Apoptosis Detection Kit from Sigma–Aldrich, and so forth. F2N12S probe kit, Violet Ratiometric Membrane Asymmetry Probe/Dead Cell Apoptosis Kit for flow cytometry, is from ThermoFisher Scientific.

6.1.1 | Protocol

Annexin V and F2N12S staining protocols are quite similar to each other like all other flow cytometry surface staining protocols but there are two important considerations: (a) annexin V binding is Ca^{2+} dependent and buffers should not contain any calcium chelating agents such as EDTA; (b) F2N12S staining is sensitive to buffers containing protein such as BSA or FBS.

Cells are washed with PBS after desired treatment conditions. Nonapoptotic or nontreated cells should be included as negative controls. For combined intracellular staining, PBS should contain 0.1% azide to stop the cellular metabolism. For the dilution factors of the staining buffers, distributor-recommended dilutions should be applied and dilution optimization should be confirmed for each cell type.

After washing step, 1,000,000–5,000,000 cells/condition or replicate are dissolved in 100 µl staining solution. For F2N12S staining, cells should be incubated for 5 min at room temperature. For annexin V staining, cells should be incubated at 2–8°C for 30 min or 10–15 min at room temperature. During the incubation, cells should be protected from light. After incubation, cells are washed, and dissolved within 100–200 µl washing buffer or the buffer that is used to dilute the staining buffer. Emission signal is measured with the flow cytometer. F2N12S signal can be obtained from the appropriate violet channels to collect the 585 and 530 nm emissions and for annexin V, appropriate channel is selected depending on the conjugated color (Demchenko, 2013) (ThermoFisher Scientific). For gating strategies, see Figure 6.

Fixation

It is possible to fix annexin V-stained cells to do permeabilization for intracellular stainings or to perform further phenotypic stainings. The fixation method should be alcohol free and aldehyde based, such as 0.5–4% formaldehyde solution, diluted with PBS. For annexin V staining, it is very crucial that binding buffers or washing buffers do not contain Ca^{2+} chelators. For the fixation step, cells are washed after annexin V staining to remove the unbound dyes. After washing, cells are fixed with formaldehyde for 15–20 min at room temperature. After fixation, cells are washed again and can be used for further permeabilization or phenotyping steps.

6.2 | Membrane permeability assays

Dying cells loose membrane integrity, whereas living cells are very strict with their membrane permeability. Analyzing membrane integrity is of great importance not only for mammalian cell culture studies to decide cell viability but also for food production research such as analyzing ethanol stress during wine production, for fertility labs to monitor the membrane integrity of sperms, and so forth (Da Silveira, San Romao, Loureiro-Dias, Rombouts, & Abee, 2002; Nagy, Hallap, Johannisson, & Rodriguez-Martinez, 2004). It is very crucial to remember that apoptotic cells keep their membrane integrity and they are resistant to exclusion dyes. On the other hand, necrotic cells can be detected with exclusion dyes (Darzynkiewicz et al., 1992). Membrane permeability assays are usually a combination of two types of dyes: one staining the dead cells and a second one staining living cells.

Membrane permeability dyes can be categorized as exclusion dyes, inclusion dyes, and monomeric cyanine nucleic acid stains.

6.2.1 | Inclusion dyes

Inclusion dyes require intracellular enzymatic functionality in addition to the membrane integrity. Cytoplasmic esterases cleave the nonfluorescent molecules and yield fluorescent compounds. Examples of inclusion dyes are fluorescein diacetate, carboxyfluorescein, and calcein (Johnson et al., 2013). Here, we describe an example protocol for calcein staining.

Reagent preparation

Calcein powder is dissolved in cell culture grade DMSO to 5 mM. Small aliquots of dissolved solution can be stored at 20°C. Like fluorometric staining reagents, calcein is sensitive to buffers and solutions containing esterases. It is not recommended to use intracellular fixation protocols together with calcein dyes.

Protocol

Cells are washed with PBS after desired treatment conditions. A total of 1,000,000 cells are prepared for each replicate and condition. For adherent cells, cells should be washed and stained prior to removing the cells from the growth surface.

Stock calcein solution (5 mM) is diluted to 10 nM final concentration using culture medium. Cells are resuspended in the diluted calcein solution and incubated for 30 min at 37°C. Cells are washed twice after incubation and measured with a flow cytometer at excitation and emission wavelengths of 488 and 520 nm, respectively (Gillissen et al., 2016). Percentage of the living cells can be calculated with one of the package software, such as FlowJo (FlowJo, LLC, USA), that is used to analyze the obtained Flow Cytometry Standard (FCS) files.

6.2.2 | Exclusion dyes

Exclusion dyes are fluorescent compounds that are excluded from the living cells and accumulated in dying cells that have leaky membranes. Propidium iodide (PI) and 7-amino actinomycin D are well-known and frequently used examples of exclusion dyes (Davey & Guyot, 2020; Johnson et al., 2013). PI stains nucleic acids and live and dead cells can be distinguished easily via very bright color of PI (Johnson et al., 2013). Here, we describe an example protocol for PI staining.

Reagent preparation

PI powder is dissolved in PBS to 2 mg/ml. After dissolving, stock solution should be protected from light and can be stored at 4°C for short-term storage up to 1 month.

Protocol

Cells suspensions are prepared after desired treatment conditions. A total of 1,000,000 cells/condition or 1,000,000 cells/replicate were resuspend in PBS containing 2 μg/ml PI final concentration (×1,000 dilution). Cells are incubated for 5–10 min, at dark and on ice. After incubation, cells were measured with a flow cytometer without washing. PI signal is obtained at 488 nm excitation/550 nm emission and analyzed with a data analysis software (Johnson et al., 2013).

PI staining is not compatible with fixation.

6.2.3 | Monomeric cyanine nucleic acid stains

Monomeric cyanine nucleic acid stains are highly sensitive nuclear staining dyes targeting double-stranded nucleic acids. A group of monomeric cyanine nucleic acid stains are provided by Invitrogen (part of Life Technologies, USA). They are compatible with fixation steps and are not excluded from the cellular membranes like other exclusion dyes. Examples of monomeric cyanine nucleic acid stains are YO-PRO, TO-PRO, and JO-PRO (Invitrogen). Alternative dyes with a wide range of emission/excision variation can be found.

In addition, there are also commercially available fixable live/dead staining kits that can be a good alternative for protocols including fixation and intracellular staining. After fixation, the cells are dead so that it is very important to start stainings first with live/dead dyes. Examples can be listed as Zombie Cell Viability Dyes from Biolegend, LIVE/DEAD Fixable Stains from ThermoFisher Scientific, Aqua Dead Cell Stain Kit from Fisher Scientific, Live/Dead Fixable Staining Kit from Promocell, and so forth.

6.3 | Mitochondria assays

Mitochondria are the powerhouses of the cells. Membrane potential of mitochondria is very critical for energy synthesis and is well regulated. Mitochondria population of a living cell is maintained by a process called "biogenesis," which is the balanced combination of fission and fusion events and is a dynamic mechanism regulated according to the status of the cell (Sedlackova & Korolchuk, 2019). It is possible to stain cells with dyes targeting mitochondria to understand the status of the cells such as apoptotic, high energy or mitochondria membrane potential (MMP) lost, and so forth.

There are several commercially available assays targeting different aspects of the mitochondria. JC-10 MMP kits are superior alternatives of poor water-soluble JC-1 dye and can be found from Abcam, Merck, and Sigma–Aldrich. In summary, JC-10 dye accumulates in mitochondria matrix of healthy cells and gives a red emission signal at 570–590 nm. Once the cells are apoptotic or necrotic, JC-10 easily diffuses into the cytoplasm and becomes monomeric JC-10, which gives a green emission signal at 520–540 nm.

MitoTracker dyes can be applied to measure total mitochondria mass or to study the changes in mitochondria mass following desired treatments. MitoTracker Green FM from Cell Signaling and fromThermoFisher Scientific are the examples of commercially available MitoTracker assays. MitoTracker Green dye stains the mitochondria of living cells but it is not dependent on the MMP. MitoTracker Green is not compatible with fixation and the signal can be obtained at excitation and emission wavelengths of 490 and 516 nm, respectively.

Another strategy of staining mitochondria is targeting mitochondrial permeability. This assay is provided by Abcam and ThermoFisher Scientific. The assays include a dye, calcein AM, and a quencher, $CoCl_2$, and both can diffuse into the cells easily. As described in the previous sections, calcein AM is cleaved by cytosolic esterases. After its cleavage, it is quenched by $CoCl_2$ in cytoplasm, but retains the mitochondria of living healthy cells. Once the cells have mitochondrial damage or apoptotic, mitochondria content is leaked and mitochondrial signal is also lost.

7 | CONCLUSION

In this guideline, the most common cell viability assays applied in research labs, namely, dye exclusion, colorimetric, fluorometric, luminometric, and flow cytometric assays, are presented. The mechanism underlying each assay and the practice of viability assessment are discussed in detail. Principles, advantages, and disadvantages of the cell viability assays are summarized in Table 1. Ideally, a cell viability assay should be safe, rapid, reliable, efficient, and time- and cost-effective, and should not interfere with the test compound. Hence, while choosing a cell viability assay, the mechanism of action of the test compound should be considered. Moreover, the type and origin of the cell line also influence the performance of cell viability assays. Considering the above, it can be concluded that the measurement of cell viability cannot be investigated satisfactorily by a single method. Therefore, it is recommended that more than one assay should be applied for the evaluation of the cell viability.

CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest to declare for this publication. [Correction added on 14 June 2021, after first online publication: The 'Conflict of interest' section was added.]

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TABLE 1 Principles, advantages, and disadvantages of cell viability assays

Assay	Principle	Advantages	Disadvantages	References
Dye exclusion assays (trypan blue, eosin, congo red, and erythrosine B)	Determination of membrane integrity	• Simple • Rapid • Inexpensive • Versatile • Require small number of cells • Erythrosine B is nontoxic, does not bind to serum proteins, and does not require incubation before counting	• Time-consuming and labor-intensive for large number of samples • Only suitable for cell suspensions, that is, monolayers need trypsinization • Counting errors may occur due to poor cell dispersion, incorrect dilution, contamination of reusable counting chambers, air bubbles, or inter-user variations • Cannot differentiate between healthy cells and viable cells that lost their function • Trypan blue is toxic to mammalian cells	Kim et al., 2016; Krause, Carley, & Webb, 1984; Ruben, 1988; Weisenthal, Dill, Kurnick, & Lippman, 1983; Yip & Auersperg, 1972; Aslantürk, 2018
Colorimetric assays (MTT, MTS, XTT, WST-1, WST-8, LDH, SRB, and NRU, CVS)	Determination of metabolic activity	• Simple • Inexpensive • Safe • Reproducible • Precise • Accurate • Rapid • Sensitive • Reliable • Applicable to both cell suspensions and adherent cells • WST-1 and WST-8 are water soluble, that is, no additional formazan dissolution step is required • WST-1 and WST-8 do not interfere with phenol red or other culture mediums • WST-8 has low toxicity	• MTT is not soluble in water, that is, additional formazan dissolution step is required • MTT may interfere with cell culture medium • MTT is highly toxic to cells • Incubation time, cell type, and cell count may affect the measurements in MTS assay • Enzymatic regulation, pH, cellular ion concentration, cell cycle variation, or other environmental factors may affect the measurements in XTT assay • Changes in intracellular metabolic activity having no direct effect on cell viability may affect the measurements in WST-8 assay • Serum and some other compounds having inherent LDH activity may affect the measurements in LDH assay • Cellular clumps or aggregates may affect the measurements in SRB assay • CVS assay is insensitive to changes in cell metabolic activity	Bopp & Lettieri, 2008; Cory et al., 1991; Decker & Lohmann-Matthes, 1988; Fotakis & Timbrell, 2006; Skehan et al., 1990; Stone et al., 2009; Strober, 2015; Tominaga et al., 1999; Präbst, Engelhardt, Ringgeler, & Hübner, 2017; Aslantürk, 2018
Fluorometric assays (Resazurin [alamar blue] and 5-CFDA-AM)	Nonspecific cleavage of a nonfluorescent compound into a fluorescent compound by cellular esterases	• Simple • Inexpensive • Safe • Rapid • Applicable to a number of cell types • Applicable to both cell suspensions and adherent cells	• Possible fluorescent interference caused by the applied test compounds • Cannot be applied to newly thawed cells • Light sensitive	Rotman & Papermaster, 1966; Altman et al., 1993; Larson et al., 1997; Schirmer et al., 1997; Ganassin & Bols, 2000; Schreer et al., 2005; Czekanska, 2011; Johnson et al., 2013; Riss et al., 2016

(Continues)

TABLE 1 (Continued)

Assay	Principle	Advantages	Disadvantages	References
Luminometric assays (ATP and real-time viability)	Correlation between a bioluminescent reaction and the effect of a tested compound	• Simple • Fast • Luminescent signal is quite stable • Signal can be measured within a few hours • Very specific • Can be applied with very few cells (>50 cells) • Real-time viability assay is the only cell viability method that allows to monitor the cell viability in real time	• Assay is prone to results with technical errors such as pipetting errors • The length of real-time viability assay measurement depends on the metabolic activity of the target cells; test compound can deplete before the end point. To avoid it, maximum incubation time is required to be optimized very carefully for each cell type	McElroy, 1947; Bulich & Isenberg, 1981; Ugarova et al., 1987; Voyevodina et al., 1990; Ugarova, 1993; Lundin, 2000; Lomakina et al., 2015; Riss et al., 2016; Aslantürk & Çelik, 2017
Flow cytometric assays				
Membrane asymmetry assays (Annexin V and F2N12S probe)	Detection of the alterations in the composition of the outer surface of cell membrane	• Fixation is possible with Annexin V staining • F2N12S probe is a small molecule, not sensitive to proteases • F2N12S staining is very fast (5 min) • For both dyes, membrane permeabilization is not required • Both dyes can be combined with other markers	• Annexin V staining is sensitive to buffers containing Ca^{2+} chelators such as EDTA • Light sensitive • False positivity following trypsin treatment	Demchenko, 2010, 2013
Membrane permeability assays (inclusion dyes, exclusion dyes, and monomeric cyanine nucleic acid stains)	Determination of membrane integrity and permeability	• Exclusion dye propidium iodide has a very bright color to distinguish live and dead cells • Monomeric cyanine nucleic acid stains are very specific double-stranded nucleic acid dyes • Monomeric cyanine nucleic acid stains are compatible with fixation protocols	• Apoptotic cells keep their membrane integrity and they are resistant to exclusion dyes • Calcein (inclusion dyes) is sensitive to buffers containing esterases • Calcein staining and propidium iodide staining cannot be combined with fixation protocols • Light sensitive	Darzynkiewicz et al., 1992; Da Silveira et al., 2002; Nagy et al., 2004; Johnson et al., 2013; Gillissen et al., 2016; Davey & Guyot, 2020
Mitochondria assays (JC-10, MitoTracker, and mitochondria permeability assays)	Determination of mitochondria membrane potential, mitochondria mass, or mitochondria membrane permeability	• Provide information about the energy status of the cells in addition to viability	• MitoTracker Green is not compatible with fixation	Sedlackova & Korolchuk, 2019; Commercial providers: Abcam, ThermoFisher Scientific, Merch, and Sigma–Aldrich

Abbreviations: CVS, crystal violet stain; F2N12S, 4′-*N*,*N*-diethylamino-6-(*N*-dodecyl-*N*-methyl-*N*-(3-sulfopropyl))ammoniomethyl-3-hydroxyflavone; LDH, lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; NRU, neutral red uptake; SRB, sulforhodamine B; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; 5-CFDA-AM, 5-carboxyfluorescein diacetate acetoxymethyl ester.

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