Applied Chemical Engineering (2025) Volume 8 Issue 4 ISSN: 2578-2010 (O)
doi: 10.59429/ace.v8i4.5582

REVIEW ARTICLE

In vitro cell-based assays to test drugs – A Review

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ABSTRACT

Cell viability is an important indicator of healthy cells. Cell viability plays a prominent role in order to determining various parameters including toxicity, as the cells may be damaged due to some physical and chemical agents. *In vitro* assays are efficient, sensitive, time-saving and were designed to replace the problems and ethical issues faced by animal models for testing the efficiency of a drug molecule. There are various assays used for determining the cell viability in eukaryotes. Assays, including Dye exclusion assays, colorimetric assays, luminometric assays, fluorometric assays and DNA-based assays involved in determining the cell viability, proliferation and cytotoxicity are discussed in this review.

Keywords: *In vitro* assays; Tetrazolium salts; Resazurin; DNA based assays

ARTICLE INFO

Received: 16 December 2024
Accepted: 19 November 2025
Available online: 11 December 2025

1. Introduction

In earlier days, diversified animals like rabbits, birds, rats, fish, guinea pigs, earthworms, fruit flies, etc. were used as a source for toxicological screening and testing of new drugs for effective development of various drug molecules and also to analyze different treatments and remedy for treating enormous infectious and contagious diseases[1-7]. They are an appropriate model for testing various biological components like antibiotics, vaccines, etc. The animal models used for research purposes either die as a result of an experiment or die due to the pain and distress caused to them during the study period. This became a highly debated topic because sacrificing animals for research purposes is considered completely unethical. Various laws have been passed against the use of animals in experiments[8-10].

To address the disadvantages, challenges, and ethical problems associated with animal models an alternative and efficient method for *in vitro* drug testing was developed[11-12]. It generally deals with the handling of cells and tissues outside the body of an animal model under specific conditions. The procedures in cell biology and

molecular biology are performed when the fundamentals of cell culture techniques are understood completely, which spreads its application in various areas like Pharmacology, medicinal chemistry, genetics, oncology, etc [13]. *In vitro* culture technique is reaching its significant success as it does not require the existence of other tissue matrix and allows the direct quantification of cells[14]. The *in vitro* models were developed for providing a repetitive, systematic and quantitative analysis of drugs. *In vitro* culture serves as a base for pharmacodynamics, pharmacokinetics analyses of drugs, high-throughput and controlled screening of drug molecules[15]. ADME properties, namely absorption, distribution, metabolism, and elimination, play a major role in drug discovery and development. *In vitro* culture generates many parameters contributing to the ADME properties than *in-vivo* methods, including stability, protein binding, drug-drug interaction potential, cytotoxicity, cell proliferation, etc[16]. *In vitro* technique allows controlled quantitative analysis and also reduces the physiological relevance by only concentrating on the microenvironment of the specific cells or tissues[17]. Assays performed in *in vitro* drug testing are explained in this review.

2. Classification of assays

2.1. Dye exclusion assays

Many assays have been used to detect the viability of cells. The dye exclusion assay is one of the efficient and simplest assays for determining the viability of cells present in the cell suspension. The principle behind this test is that live cells with an intact cell membrane do not take up the dye, whereas dead cells take up the dye easily. Eosin, Trypan blue, Congo red, and Erythrosine B are some of the dyes used in the Dye exclusion assay[18-20]. Several studies have suggested that fluorescent dyes are more effective and reliable markers of cell viability than conventional dyes. Since fluorescent dyes remain stable after being taken up by cells, viability can be measured over a period of time. Propidium iodide and other DNA-intercalating dyes, including trypan blue, are used to estimate cell membrane transport properties. These dyes are reportedly taken up by non-viable cells that have lost their membrane permeability barrier or dye exclusion capability[21]. The degree of nuclear membrane disruption can influence the strength of orange fluorescence seen in non-viable cells. As a result, intercalating dyes are ideal for detecting non-viable cells[22].

2.1.1. Trypan blue dye exclusion assay

Trypan Blue is one of the earliest and traditional methods used in the dye exclusion test. The weight of Trypan Blue is approximately 960 Da and is a negatively charged azo dye. Because of its nature, it can only penetrate into certain cell membranes, but as soon as it enters the cell, it binds and interacts with the intracellular proteins available nearby and produces the blue colour (**Figure 1**). It is a direct quantification of dead and live cells[23-26]. The Trypan Blue (TB) method is a popular cytotoxicity assay in experimental studies in which dead cells absorb TB into the cytoplasm due to a loss of membrane selectivity, whereas live cells remain unstained. Optical Microscopy is used to determine the relative number of dead and live cells by counting the number of stained (dead) and unstained (live) cells in a Neubauer Chamber[27]. Because of the long run time and the requirement of extensive microscope; this traditional TB exclusion assay can produce low-precision results when used for a large number of samples. TB-protein complexes have been shown to produce fluorescence in previous studies[28,29]. The principle behind this assay is that the cell suspensions are subjected to TB staining the viable cells exclude the dye and have a clear cytoplasm and the dead cells take up the dye and have a blue cytoplasm[30-32]. This method offers several advantages: it is less expensive, simpler, and provides detailed information about membrane integrity. Additionally, it is safe when used for short periods of time[33-35]. This method also contains some disadvantages like less amounts of samples can only be processed at a single time as haemocytometer is used for cell counting, therefore the error percentage is approximately equal to 10%. It cannot distinguish between live healthy cells and unhealthy cells that have lost their function[19]. The sensitivity of the assay is not well specified. But Nowak et al. [36] reported that each

well in a 24-well plate is filled with 1,30,000 cells in a volume of 1 mL. After the halt of the trypsin reaction, the cell suspensions were centrifuged which was followed by the addition of 10 µL 0.4% trypsin and the viability of cells were determined by counting the cells in haemocytometer. Bellamakondi and team seeded 2 × 10^5 cells per mL and after centrifugation, 0.5% of trypan blue dye was added and they reported the death count of cells by observing it under haemocytometer[37]. Another group of researchers reported that 1× 10^6 cells were used for performing the trypan blue dye exclusion assay[38]. Although the TB exclusion assay has long been the standard approach for determining cell viability, it has several disadvantages. Because of the toxic nature of tuberculosis, the time between cell staining and counting is restricted[39]. Second, since tuberculosis binds to cellular proteins, it can bind to non-specific cellular objects, especially in clinical and primary cell samples. Finally, there is currently no standardisation of TB concentration for cell viability calculation. Finally, manual counting with a hemacytometer under a simple light microscope is used to rule out tuberculosis. This can be time-consuming and subject to operator variance[23]. In human Retinal Pigment Epithelial (RPE) cells, the effects of trypan blue on cell viability and gene expression were investigated by Kwok et al.[40], which showed that at higher concentrations, trypan blue can cause toxicity in cultured RPE cells, as evidenced by a decrease in cell viability and changes in the expression of apoptosis-related and cell cycle arrest genes. A one-minute application of 0.06 mg/mL trypan blue appeared to have little effect. Cell viability of biogenic synthesized nanoparticles from chitosan was evaluated for its cytotoxicity using trypan blue dye exclusion assay which showed that the cytotoxic efficacy of the synthesised AgNPs was revealed in a dose-dependent manner using the trypan blue , with viable cells appearing colourless and non-viable cells appearing blue[41].

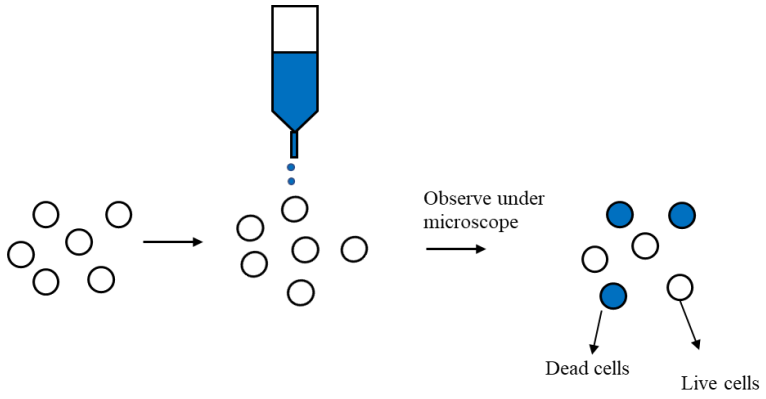

Figure 1. Trypan blue Dye exclusion assay

2.1.2. Erythrosine B dye exclusion assay

Erythrosine B dye is an integral dye but compared to trypan blue dye, it is not very prominent and is used rarelyin cell counters. For several gram-positive and negative bacteria, Erythrosin B (EB), a visibly red dye with fluorescent properties, serves as a critical dye. For all bacteria tested, EB performed at a similar concentration, and incubations were as short as 5 min. Because of the spectral properties of EB, a variety of experimental methods can be used to quickly image and/or quantify dead bacterial cells in a community. EB is the first widely available colorimetric viability dye for bacteria, making it a cost-effective option for academic and industrial researchers[42]. This cherry pink dye is not permeable into the cell membrane but it is widely used as a food additive[43-46]. The principle behind this assay is similar to the trypan blue assay as it only stains the cell that are dead and the viable cells remain unstained. This stain is considered to be biosafe and an alternate use for trypan blue. At single concentration, it can work rapidly for various bacterial species[42,47] (**Figure 2**). It has many advantages including versatility, being inexpensive, biosafe, but it consumes more time, is labor intensive, and possess possibility of contamination when reused[19,48]. The

sensitivity of the assay has not been specified; further studies are required to determine its accuracy and reliability. The EB assay has several drawbacks, the most notable of which is that it is not a process suitable for automatic counters. As a result, multiple errors can occur due to human errors and the use of reusable cell counting chambers. There are many studies using the Erythrosine B dye exclusion assay since non-invasive and non-harmful assays for cell viability are needed for research and innovation in the regenerative medicine field, drug development studies, genetic engineering, single-cell analysis, microbial food culture, and other biotechnologies[49]. Erythrosine photo-bleaches in dilute solution using first-order kinetics, with the rate of bleaching being highly dependent on the rate of photon absorption and the concentration of dissolved oxygen. Self-catalysis appears to enhance this inherent bleaching at higher concentrations of erythrosine and for longer exposure periods, according to kinetic studies[45]. 11 dentin discs were divided into 2 halves, sterilized and demineralized for 30 mins. After the application of erythrosine, the depth of penetration of erythrosine into dentin was observed under Photoacoustic Spectroscopy. The erythrosine penetration caused decay in dentin[50]. This approach was used to examine many natural food pigments and to see whether monascus pigment or anthocyanin pigment was present. Anthocyanin pigment acts as good dye exclusion viability indicator for *Euglena gracilis* testing[51].

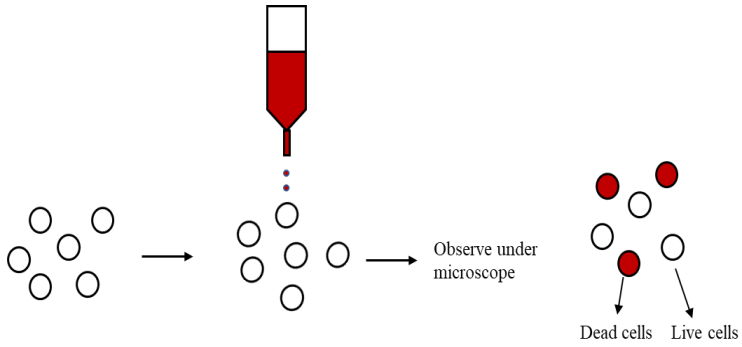

Figure 2. Erythrosine Dye exclusion assay

2.2. Fluorometric assays

2.2.1. Alamar Blue assay

Alamar Blue assay have been used for approximately 50 years to determine cell cytotoxicity and cell viability. It is also used for various environmental and biological applications and is a bio assay used for quantifying the cell proliferation in fungi, bacteria, animal and human cell lines[52]. The most important ingredient is resazurin[53] which is a non-fluorescent, non-toxic, non-radioactive, water-soluble, stable and permeable through all the cell membranes. Alamar blue is not involve in normal electron transfer and is involved in intermediate electron transfer. It is a blue colour dye in oxidized form which accepts an electron and reduces to pink colour resorufin which is measured colorimetrically. The change in colour indicates the presence or absence of viable cells[54,55]. It can also be reduced by several other enzymes like mitochondrial reductase, flavin reductase, diaphroases present in mitochondria. It has high sensitivity, no cell lysis, accurate time course quantification and can be used in all different kinds of cell models[56,57]. Cells should not be incubated for a longer time with resazurin dye as it results in a secondary reduction of pink resorufin (**Figure 3**). The resazurin can be stored at room temperature, 4 ℃ or -20 ℃, depending on different manufacturers but lower temperature attributed to high sensitivity. Both resazurin and resorufin are sensitive to light, hence prolonged exposure to light affects the sensitivity[58-60]. It can involve 50 to 50,000 cells in a 96-well plate[58,61]. 1×10^6 cells were added as 200 µL per well in a 96-well plate for performing Alamar blue assay[62]. 10 to 20,000 cells per well of fibroblast cells were seeded in a 96-well plate by Voytik-Harbin et al.[63], for evaluation of cell growth and survival using Alamar blue staining. 2.5×10^6 cells/mL of

promastigotes of *L. major* MRHO/IR/76 vaccine strain was seeded along with alamar blue stain for screening anti-leishmanial drugs like Pentostam, Amphotericin B and Paramomycin[64]. 78 clinical isolates from the Golestan Regional Tuberculosis Reference Laboratory (GRTRL) were detected using the Microplate Alamar Blue assay (MABA), and the findings were compared to the proportion process. Between the Alamar blue-rifampicin and the proportion system, high accuracy was discovered. In reality, alternative assays for the detection of RMP-resistant tuberculosis in low-income countries include the fast and Low Microplate Alamar Blue assay[65]. The toxic effect of a mixture of Alamar Blue and Au(III) on *Trypanosoma brucei* was investigated, and it was discovered that the IC_{50} value for aqueous Au(III) was 3.8 x 10^{-7} M, while the IC_{50} value for Pd(II) was 1.3 x 10^{-5} M. However, neither Alamar Blue nor the ion Au(III) had a noticeable toxic impact on the parasite. The toxicity of a mixture of Alamar Blue and Au(III) ion in mice infected intraperitoneally with 4.0 x 10^4 trypanosomes was also tested *in-vivo*. For the mixture of Alamar Blue and Au(III) ion, the mechanism of DNA cleavage and toxicity *in vitro* tests are discussed by Habib et al.[66].

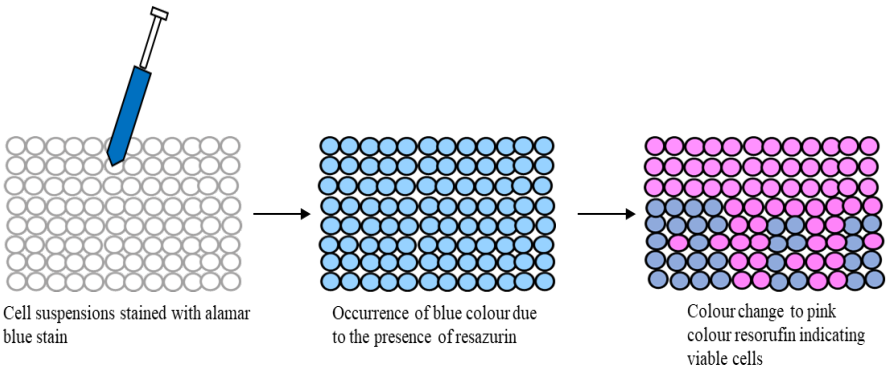

Figure 3. Alamar Blue assay

2.2.2. CFDA-AM assay

5-Carboxyfluroscein Diacetate-Acetoxymethyl Ester (CFDA-AM) is another dye used for determining cell proliferation[19]. Another fluorogenic dye is used in cytotoxicity experiments to determine plasma membrane integrity. It is a non-toxic esterase substrate that can be converted from a membrane-permeable, non-polar, non-fluorescent material to a polar, fluorescent dye, carboxyfluorescein, by non-specific esterases in living cells (CF). The cell's conversion to CF indicates the plasma membrane's integrity, as only an intact membrane can maintain the cytoplasmic milieu required for esterase activity[67]. Cytosolic Esterase substrates are used for understanding the metabolic activities of the cell by acting as a viable dye. The dye CFDA measures the integrity of the cell membrane through enzymatic activities. The conversion of non-polar, non-fluorescent components into a polar and fluorescing carboxyfluroscein by the non-specific esterase of living cells determines the integrity of cytoplasmic membrane and plasma membrane (**Figure 4**). This assay allows high sensitivity even for a smaller number of cells[68,69]. Since CFDA has more negative charges, it is best preserved in cells. The carboxyfluorescein diacetate acetoxymethyl ester of CFDA (5-CFDA-AM), which is electronically neutral and can be loaded into cells at lower concentrations than CFDA, is one of the most widely used derivatives. This is a lipophilic substrate that is moderately permeant to most cell membranes, similar to the other CFDA derivatives[70]. Intracellular non-specific esterases cleave diacetate from the molecule, releasing fluorescent carboxyfluorescein (CF), which is effectively retained by live cells with intact plasma membranes. Using widely used fluorometric cell viability assays based on chemical reagents, the acute toxicity of the manufactured Pt NPs and Pt ions against two separate cell lines, EPC and BF, was investigated. First, statistically significant differences compared to control samples were observed with the exposure of 50 and 75 mg/L of Pt ions and Pt NPs, respectively, based on CFDA-AM assay findings, which

is a marker of membrane integrity. This decrease means that the integrity of the cytoplasmic (cell) membrane has been reduced[71]. The CFDA-AM assay was used to assess cytotoxicity in two fish hepatoma cell lines and one mammalian hepatoma cell line, the plasma membrane integrity was found using CFDA-AM assay[72].

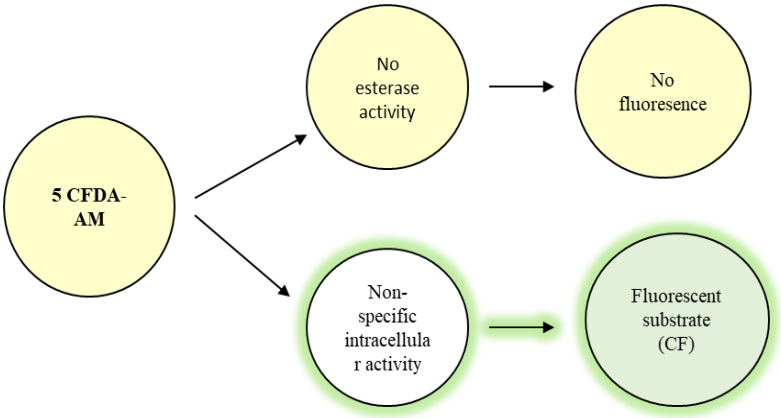

Figure 4. CFDA-AM assay

2.3. Luminometric assays

2.3.1. ATP assay

ATP is an essential source of life as it accelerates various chemical reactions in our body and also serves as a main source in signal transduction, chemical signalling and energy metabolism[73,74]. Adenosine Triphosphatase (ATP) assay is a bioluminescence assay and is directly proportional to viable biomass present in the sample, i.e., it can quantify the accurate number of active biomass present in the sample[75]. It involves reduction of luciferin to oxyluciferin, which happens in the presence of Mg^{2+} ions and ATP, yielding signals. It is widely used as a 1536-well plate method as it can detect only about 10 cells per well[19]. ATP assays uses bioluminescence as an indicator to find the presence of alive cells and are used to measure the functional integrity of all the viable cells as all the viable cells require ATP (**Figure 5**). It has an enormous dynamic range of 0-7,50,000 RLU (Relative Light Units) in RPMI 8226 cells, but is not limited to only carcinogenic cells[76]. Monitoring of drinking water employs the use of ATP assays[77], but the sensitivity of ATP assay is not well specified and still needs more studies. Santangelo et al.[78] Seed about 100 µL of *E. coli* culture in each well of 96 96-well plate and ATP assay was performed using ATP kit and the software used, provided the luminescence signal intensity of the ATP concentration in counts per second. Several methods have been applied in order to improve the sensitivity of the assay. *Leishmania promastigotes* at logarithmic phases were seeded in 96 96-well plate in variations of concentration from 1.10^4 to 4.10^7 parasites/mL with a final volume of 100 µL per well. The quantitation limit was found to be 52 (Qlx) with a lower detection limit of 3.7 which was lower than that of the MTT assay[79]. Some methods that involved enzymatic conversion of AMP and ADP to ATP are a long process that takes around 1 h for incubation; therefore, a simple method was designed by Lee et al.[80], to increase the sensitivity of the ATP assay by increasing the production of ATP from bacteria, and subjecting it to heat treatment and bioluminescence was calculated using a luminometer and found that ATP production from bacteria increased. Among the other assays, ATP assay has greater sensitivity, is fast, reliable and can be performed in a short amount of time[60]. A nanosensor for intracellular ATP assay was developed based on a polyimidazolium brush (PimB)-modified nano-pipette. This sensor demonstrates good selectivity, high spatiotemporal resolution, and excellent stability. The rationally tunable property of ion transport at a confined nanopipette, as well as the unique supramolecular interaction between polyimidazolium and ATP, allows the selective assay of ATP even in complex intracellular reactions. The nanosensor has been shown to be useful for *in-situ* monitoring

of chromaffin cell basal ATP levels in wild-type and DJ-1 knockout mice, as well as real-time monitoring of intracellular ATP metabolism[81]. Rapid detection (20 min) of *Streptococcus agalactiae*, Group B *Streptococcus* (GBS), using on-chip magnetic isolation of GBS based on immiscible filtration assisted by surface tension (IFAST), followed by detection of the isolated GBS using an ATP bioluminescence assay. Up to 80% of GBS cells were extracted from spiked artificial urine samples, with linear responses of bioluminescence signals from isolated cells ranging from $2.3x10^2$ to $9.1x10^5$ CFU mL, indicating great potential for point-of-care detection of pathogenic bacteria in pregnant women's urine samples[82].

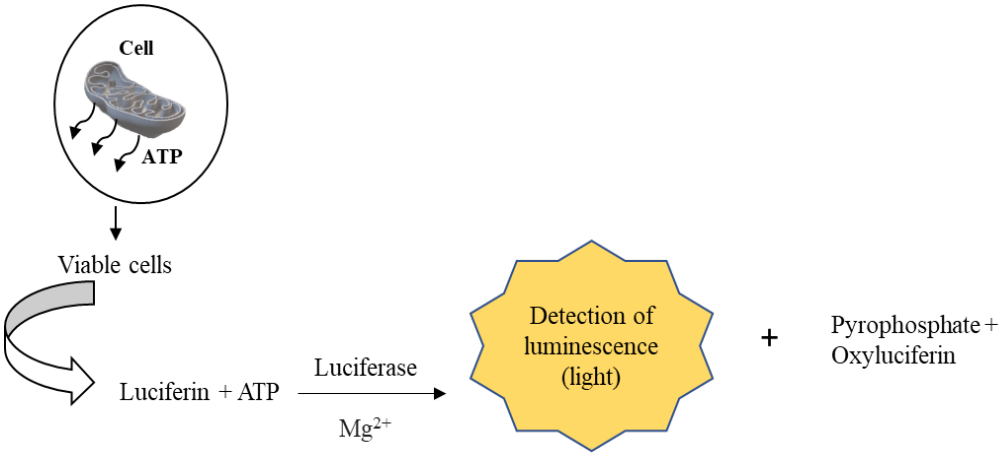

Figure 5. ATP assay

2.3.2. Real time viability assay

Radiational end point assays have significant disadvantages as compared to real-time continuous monitoring of cellular processes. A detailed representation of changes in live cells over the course of an experiment offers knowledge about the cell's biological status and informs decisions about treatment timing and the use of other functional endpoint assays[19]. Duellmann et al.[83] identified a non-lytic, homogeneous bioluminescent assay for real-time cell viability measurement. The health of the cells was tracked for 72 h from the same test samples, differentiated between different cell development, and the drug mechanism of action by examining time and dose-dependent drug effects was investigated. The real-time measurements also enabled researchers to detect cell death quickly, compare drug potency to efficacy, and distinguish between cytostatic and toxic drug effects. A new method developed in recent days to determine the viability of cell count in real time employs the use of luciferase enzyme from a marine shrimp along with a pro-substrate. The engineered luciferase and the pro-substrate were added directly to the culture medium as a reagent for this assay. The viable cells with active metabolism convert the pro-substrate into substrate and it is diffused into the culture medium, where it is absorbed by the engineered luciferase enzyme to produce the luminescent signal (**Figure 6**). There are two modes of real-time viability assay: continuous mode assay and endpoint mode assay[60,83]. A naturally secreted luciferase from the marine copepod *Metridia longa* was recently described and cloned. Metridia Luciferase (MLuc), a 23 kDa enzyme, is appealing as a reporter because it catalyses a basic bioluminescent reaction that only includes coelenterazine and oxygen. The end result is a blue bioluminescent signal (max = 480 nm) that can be observed in traditional luminometers with high sensitivity over a broad dynamic range. Additional secreted luciferase genes, including MpLuc1 and MpLuc2 from the related *Metridia pacifica* copepod and Gaussia Luciferase (GLuc) from the *Gaussia princeps* copepod, have been cloned since this discovery[84]. All of these secreted proteins show great potential as reporters for non-end point monitoring of biologic processes at high throughput. The sensitivity of this assay was not well defined, and more research is needed in this area[85].

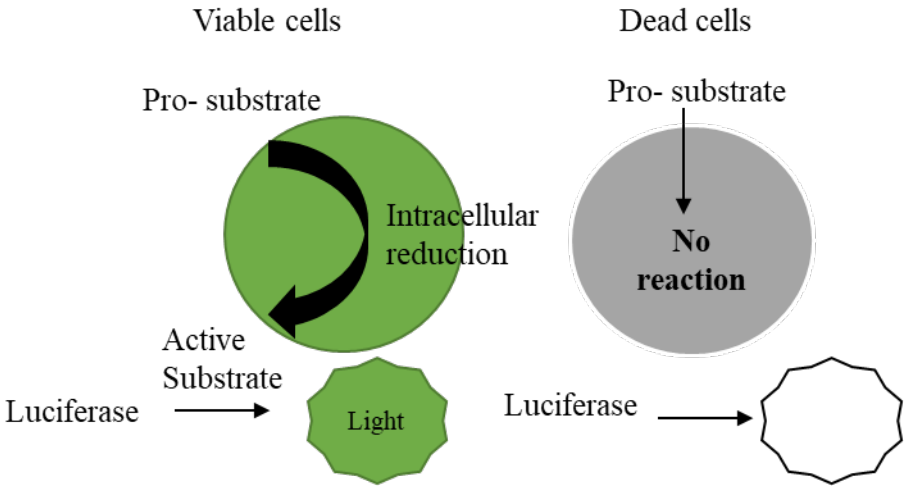

Figure 6. Real time viability assay (Inspired from Riss et al. [60])

2.4. Colorimetric assays

2.4.1. MTT assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay aim is to quantify viable cells in a reasonably high throughput (96-well plates) without the need for complicated cell counting. As a result, the most popular application is to assess the cytotoxicity of a variety of drugs at various concentrations. MTT assay is one of the most popular and versatile tetrazolium salt-based assays used nowadays. In this assay, by the action of mitochondrial reductase, a water-soluble dye is converted into insoluble formazan, a purple colour. The MTT assay works on the assumption that mitochondrial activity is constant for most viable cells, so an increase or decrease in the number of viable cells is proportional to mitochondrial activity. The conversion of the tetrazolium salt MTT into formazan crystals, which can be solubilized for homogeneous calculation, reflects the mitochondrial activity of the cells. As a result, any increase or decrease in viable cell number can be detected by measuring formazan concentration as reflected in optical density measured at 570 nm following formazan solubilization[86]. Approximately 10^6 cells can be accumulated in a single well[87,88]. MTT, due to the net positive charge and lipophilic nature, cannot pass through the membranes of the cell and hence gets reduced in the viable cell present by the action of some of the mitochondrial enzymes like oxidase, oxidoreductase, dehydrogenase, peroxidase, NADPH, NADH etc. and leads to the conversion of formazan (**Figure 7**). A crystalline and a solid product is formed in an assay containing MTT, which grows sharply and eventually affects the integrity of the cell membrane, leading to cell death. Due to this, the formation of formazan is stopped and this indicates the end point of the reaction. Cell viability is determined after the endpoint [89,90]. The colour change is measured using a spectrophotometer. Under optimised and stable conditions, the absorbance measured is directly proportional to the total number of viable cells[91]. The cells that are viable, healthy and rapidly growing shows a high rate of conversion of MTT to formazan while the cells that are dead show negligible reduction of MTT[92]. This can be scaled up to 500 to 10,000 cells per well in 96 96-well plate format and it has linearity for around 10^6 cells[87]. Van Tonder et al.[93] reported that the MTT assay has less sensitivity for cells below 1000 cells per well. The incorporation of a growth cycle after the drug has been removed is a key feature of the assay. This helps cells to rebound from the drug's effects or die, and it also prevents the drug from interfering with MTT Reduction[94]. The research conducted by Samrot et al.[95] involved evaluating the anti-cancer activity of

SPIONs, latex-coated SPIONs, and drug-loaded nanoconjugates against both normal fibroblast L929 and breast cancer MDA-MB-231 cell lines using MTT assay.

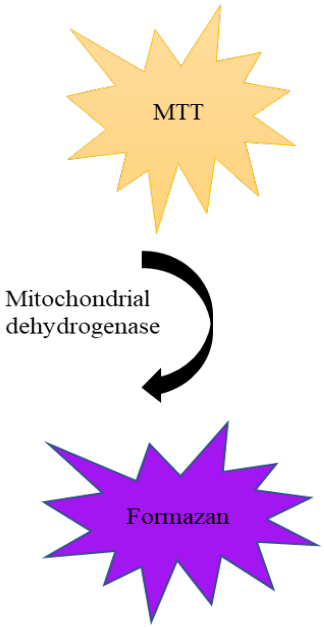

Figure 7. MTT assay

2.4.2. MTS assay

The MTS *in vitro* cytotoxicity assay has been found to be a useful tool for determining cell viability in a variety of studies. The ease of use, precision, and rapid indication of toxicity are the key features of this assay. If it can be shown that this assay has reasonable sensitivity and specificity, it may be a valuable tool in human health risk assessment. This is especially relevant when exposure to unknown chemical compounds necessitates rapid identification and assessment of toxic effects[96]. NAD(P)H-dependent oxidoreductase enzymes expressed in mitochondria can convert tetrazolium to coloured formazan in live cells, 1–7 proportional to the metabolic activity of mitochondrial enzymes. The amount of formazan emitted can be calculated using a spectrophotometer, which can then be used to estimate cell numbers[97]. The MTS assays are based on the measurement of a mitochondrial enzyme responsible for the conversion into formazan. Electrons generated by mitochondria to its formazan products are responsible for the reduction of MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H tetrazolium)[98]. The electron acceptor reagent, phenazine methyl sulfate, is used along with the tetrazolium for penetration into viable cells and gets reduced in the cytoplasm to form formazan (**Figure 8**), where this formazan is dissolved using DMSO or DMF and colorimetrically evaluated[60]. This MTS tetrazolium reagent can be reduced by the cells that are viable and generate formazan product, which is directly soluble in cell culture medium[99,100]. The MTS reagent avoids the liquid handling step as there is no need to add another reagent to the plate for formazan solubilization. The negative charge of formazan limits the cell permeability of tetrazolium[101]. Sensitivity of the assay needs more study.

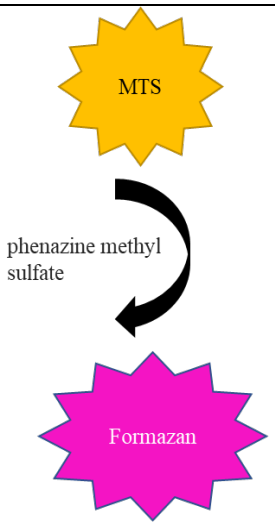

Figure 8. MTS assay

2.4.3. XTT assay

The mitochondrial activity of the metabolically active and healthy cells is measured using a semi-quantitative colorimetric method: XTT assay. It is based on the principle that a yellow colour (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) tetrazolium XTT salt is converted into an orange colour formazan, which is water soluble in nature[102-104]. XTT is a negatively charged tetrazolium salt, that uses an electron acceptor that facilitates the transfer of electrons from the plasma membrane or cytoplasm for the conversion of XTT into water-soluble formazan. This electron acceptor generally increases the sensitivity of the assay. The procedure is mostly similar to MTS assay[60]. Figure 9 indicates the XTT assay. The XTT assay is used to measure the cell's pyridine nucleotide status (NADPH and NADH as the parameters to measure the metabolic activity of cells)[105]. The sensitivity of the assay is not well specified. Nowak et al.[36] seeded 15,000 HepG2 cells at a total volume of 100 µL per well in a 96-well plate and performed the XTT assay. Chiyomaru et al.[106] seeded around 3000 cells in a 96-well plate and cell viability was determined using XTT assay. Human osteoblasts cells were seeded in 96 96-well plate at a density of 10,000 cells per well and mitochondrial dehydrogenase activity was observed using the XTT assay. Despite the convenience and speed of the assay, problems related to inter- and intra-species variations are recorded.

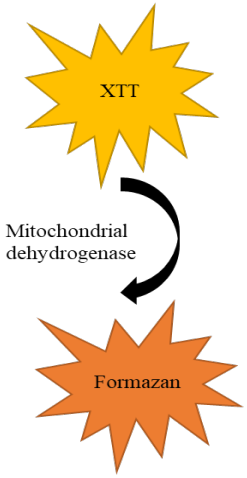

Figure 9. XTT assay

2.4.4. LDH assay

The lactose dehydrogenase leakage assay is generally a protein assay. It is an enzyme that is generally present in the cytoplasm of the cells[107]. The viability of cells is assessed by the measurement of lactate dehydrogenase enzyme present in the external medium. Cell death is indicated by the dropping level of intracellular lactate dehydrogenase enzyme and its penetration into the culture medium. As a result of this process, the membrane of cells gets damaged which eventually leads to the death of cells. The principle behind this assay is that in the presence of LDH lactate and is converted into pyruvate and parallel, NAD is reduced to NADH and the absorbance is measured at 340 nm. The conversion of yellow tetrazolium salt into red water-soluble formazan indicates the number of dead cells in the medium[61,108-110] (**Figure 10**). Membrane integrity is determined by the amount of LDH present in the extracellular medium[111]. It is considered to be 25 times cheaper than other assays[112]. The LDH kit available can be used with various cell types and is fast, reliable and simple. Mostly Triton-X 100 is used as a positive control for the determination of LDH released from the cells[19]. Cell dilutions are prepared in a way that 0, 5000, 10,000 and 20,000 cells were diluted and 100 µL is added in 96 96-well plate and the percentage of cytotoxicity is calculated using LDH release[61].

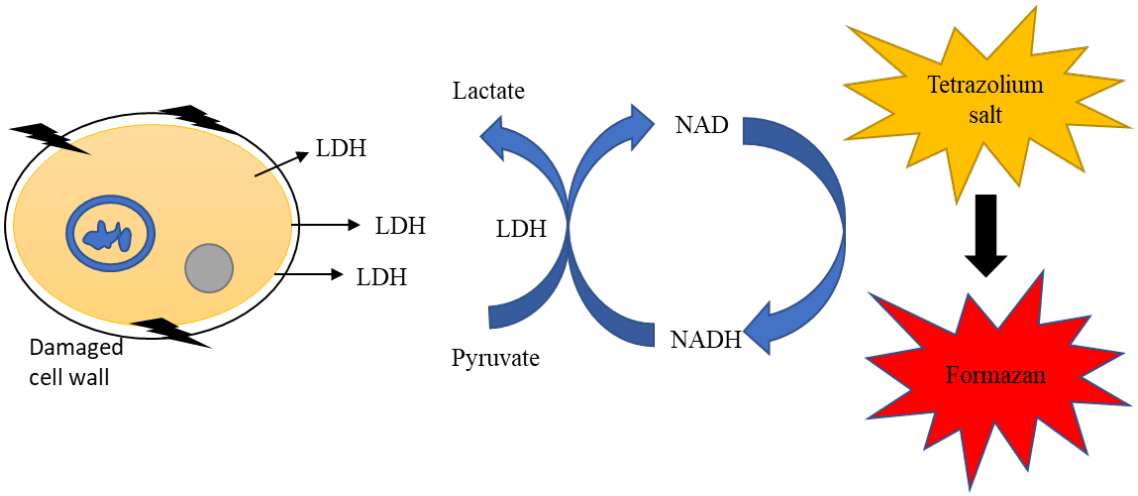

Figure 10. LDH assay (Inspired from Forest et al. [113])

2.4.5. SRB assay

Sulforhodamine B (SRB) is used for determining the cell density by assessing the proteins present in the cellular level[114] and for detecting cytotoxicity in the cells [93]. The principle behind this assay is that the SRB dye binds with the cellular proteins at mild acidic conditions and then excess dye can be removed at basic conditions. The dye bound with proteins are used to calculate cell viability, which can be measured spectrophotometrically by calculating the absorbance at 510 nm[115,116]. SRB dye is generally a pink amino xanthene dye that forms an electrostatic complex with amino acids that is basic in nature at acidic conditions and can be retracted at basic conditions. The more cells, the more stain uptake after cells are lysed by fixing them on a slide. The SRB dye gives a brighter red colour and greater absorbance. It is a rapid, sensitive, stable and easily reproducible assay. It has a good signal-to-noise ratio and good linearity among other formazan-based assays[117]. This can detect around 5,000-50,000 cells per well[118]. SRB stain is a kind of protein stain and is used for *in vitro* chemosensitivity testing. SRB assay has a better linearity and reproducibility and is more sensitive compared to MTT assay[114] (**Figure 11**).

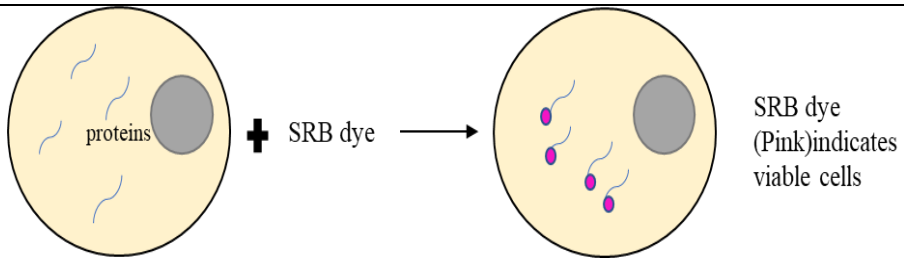

Figure 11. SRB assay

2.4.6. NRU assay

Neural Red Uptake assay is a cell viability assay that quantifies the cytotoxicity induced by xenobiotics and is generally calculated by the amount of dye that binds to the lysosome (**Figure 12**). It is a Eurhodine, a weak cationic dye that enters the cell at physiological pH by means of non-ionic diffusion[119,120]. After intracellularly entering the lysosome, acidic pH is generated by the proton gradient and the dye becomes charged. The characteristic like membrane permeability and lysosomal activity enables to identify the viable, healthy, damaged and non-viable cells[19,121]. NRU assay is generally used for accessing the hazard for *in vitro* toxicology application. Two different cell concentrations were added (3,900 cells/m^2 and 62,500 cells/m^2) in a 96 well plate and NRU assay was performed to calculate the cell viability[122]. 2×10^5 cells per well were seeded in a 96-well plate for performing NRU assay which was found to be sensitive and provided accurate response after exposing the coelomocytes primary culture cells to metals at *in vitro* condition [123]. 3×10^3 cells per well was seeded in a 96-well plate for analysing the cytotoxicity of lignan grandsin using neural red uptake assay[124]. Accurate sensitivity of NRU assay needs more study.

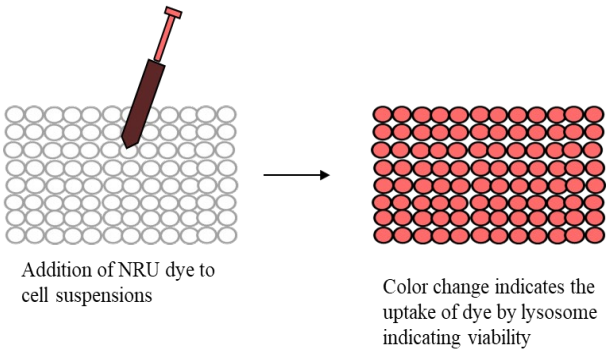

Figure 12. NRU assay

2.4.7. CVS assay

Crystal Violet assay is also known as hexamethyl pararosaniline chloride or Gentian violet stain. Crystal violet stain is a type of microbiological stain that is used to identify bacteria and cell numbers in monolayer culture by the principle of absorption of dye. With more and more improvements nowadays, crystal violet dye is used for the detection of cell death and cytotoxicity caused by certain toxins or drug compounds[125]. Crystal violet staining is the direct quantitative measure of the masses of DNA in a living cell [126]. The crystal violet dye binds with protein and DNA in a cell and the viability count is determined if the dye completely binds and in case of dead cells, the cells lose its adherence from group of cells stained using crystal violet dye (**Figure 13**)[127]. This assay is generally used to identify growth rate reduction by estimating the cells colourimetrically [128]. Usually, cells ranging from 1 to 2×10^4 are seeded in 96-well plate for achieving 40 to 50% confluence. But initial confluence depends upon the cell lines, cell size and cell proliferation[127]. The crystal violet assay can be used for biofilm quantification. The advantage of crystal

violet assay is that it can measure the total count of biofilm but cannot measure the functional biofilm. Crystal violet stain can stain both Gram-positive and Gram-negative cells[129,130].

Figure 13. CVS assay

2.5. DNA based assays

2.5.1. AO/EtBr staining

Acridine orange and Ethidium bromide staining can penetrate into the normal cell membrane and it can be observed as green fluorescence, whereas, in apoptotic cells, due to nuclear shrinking, it is observed to have orange fluorescence and necrotic cells are found to have red fluorescence due to loss of membrane integrity[131]. It is used to evaluate the nuclear morphology of apoptotic cells. Acridine orange dye can stain both viable and non-viable cells, but ethidium bromide dye can stain only the cells for which the membrane integrity is lost[132,133]. It is used to differentiate between actively proliferating cells and inactive cells[134]. This process involves a principle in which Acridine orange stains all cells, including viable and non-viable ones, producing green fluorescence. Then, Ethidium bromide (EtBr) is added, which only stains dead cells, diminishing the green fluorescence produced by Acridine orange. Viable cells are indicated by a green colour, while non-viable cells appear yellow, red, or orange (**Figure 14**)[135]. Acridine orange/ethidium bromide staining of Hep G2 cell lines was conducted using *Terminalia Catappa* L. gum-based curcumin-loaded nanoparticles to detect apoptosis. The results, indicated by green fluorescence, concluded toxicity[136].

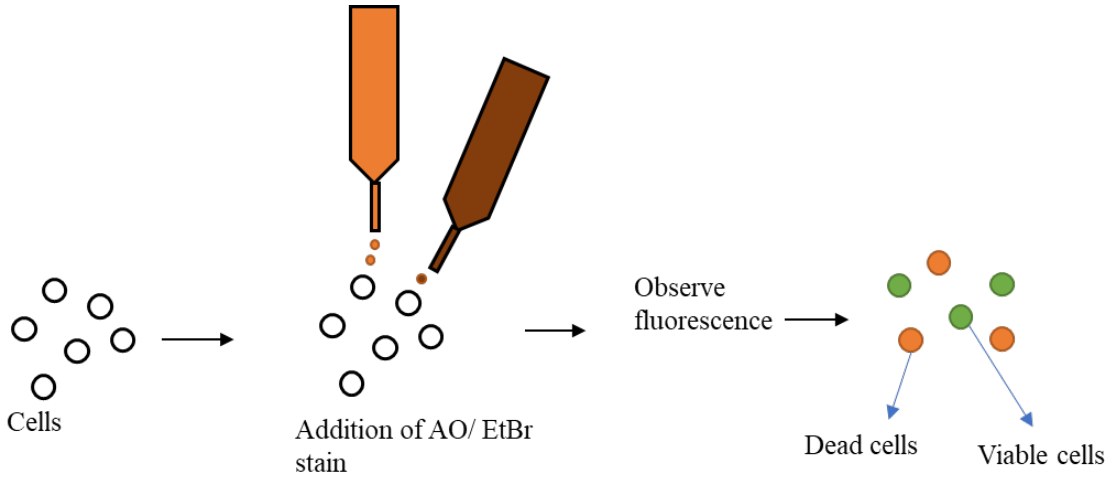

Figure 14. AO/EtBr staining

2.5.2. AO/PI staining

AO/PI staining is used for high-throughput cell viability, cell concentration and consistency. It makes use of 96 to 384 well plates and the sample can be analysed in about 7 mins and improving the efficiency of high throughput assays[137]. Acridine Orange dye stains both dead and viable cells, but the propidine iodide stain stains only the dead cells. Apoptosis is indicated by chromatin breakdown. This involves the mechanism in which cells after being stained by AO, green fluorescence is diminished by the addition of PI stain as it binds with DNA and stains only the dead cells because PI can enter only inside the cell that has damaged the cell wall (**Figure 15**)[138-141].

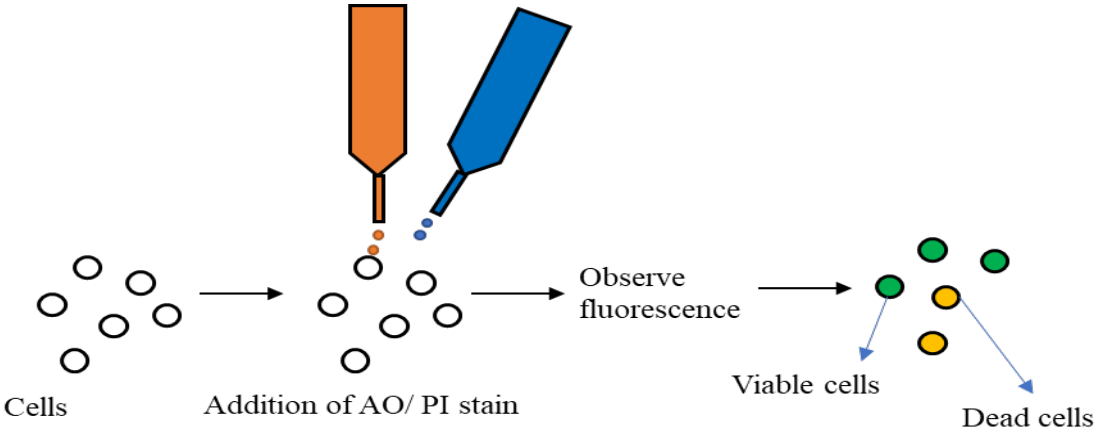

Figure 15. AO/PI staining

2.5.3. COMET assay

COMET assay is also known as single cell gel electrophoresis, which has attracted many researchers over a past decade due to its ability to find damage in DNA, biomonitoring human, applications in genotoxicity, with main research in assessing damage in DNA and its repair. This assay has been of use due to its simplicity, versatility, sensitivity, economy and speed. High salts and detergents are used for lysis for immobilisation of DNA for electrophoresis after embedding cells in agarose. COMET assay is mostly used for animal culture in the form of isolated or present in culture (**Figure 16**)[142,143]. Calibration of assay is done and for measuring the total DNA damage, few doses of ionising radiation are passed and the Gy equivalents are used to measure the comet score. 0.31 breaks per 10^9 Da of DNA was introduced when 1 Gy of ionising radiations were passed, which is equal to the breakage of 1000 diploid mammalian cells[144]. This method attracted many researchers because of three reasons: 1000 cells were only required, no labelling with radioisotopes and between the cells of same population variations in response to DNA-damaging agents were determined[145]. This assay can be applied for tissue or cell lines from which differentiated cells were obtained from single cell suspension. It has its applications *in vitro* , *in-vivo* and *ex-vivo*[146]. This assay can only tell the break in the strands and alkali label sites. The major disadvantage is the checkpoints in the cell cycle cannot be identified by this assay[147]. This assay has the highest sensitivity and can detect even lower levels of DNA damage in cells. It requires a smaller number of cells per sample and is flexible, inexpensive and requires a short span of time. This is a high-throughput screening assay and has the ability to distinguish between chromosomal damage caused by genotoxicity and cytotoxicity[148,149].

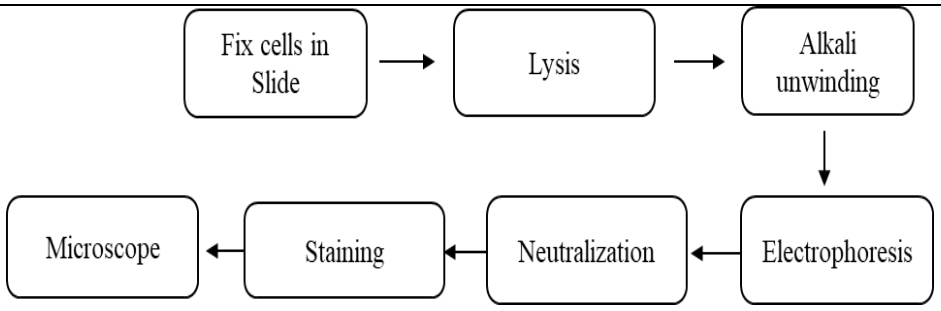

Figure 16. COMET assay

2.5.4. Tunnel assay

During the last stages of apoptosis, cells undergo a large amount of DNA degradation, which can be measured by Deoxynuleotidyl Transferase (TDT) dUTP Nik End Labelling (Tunnel assay). The main principle of this assay involves the identification of blunt ends of double-stranded DNA, which are independent of templates. TDT (Terminal Deoxynucleotidyl Transferase) and dUTP (Deoxyuridine Triphosphate) are labelled and added by the enzyme to the 3' hydroxyl terminal of double-stranded DNA. This activity can be observed using histochemical techniques (**Figure 17**)[150]. This assay can be used for clinical samples such as solid tumours, leukaemia, lymphoma, etc.[151]. Not only apoptotic cells, even necrotic cells, DNA repair, cells damaged by mechanical forces and also cells that undergo gene transcription can be detected by the Tunnel assay and is used to stain the cells that undergo apoptosis and programmed cell death and it can also stain the cells that undergoes DNA repair[152].

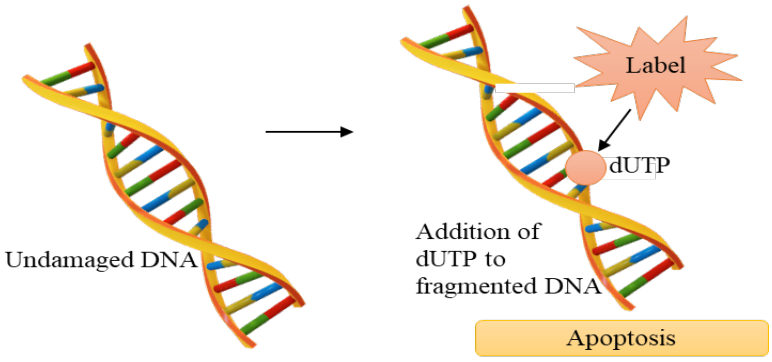

Figure 17. Tunnel assay

2.5.5. Caspase assay

Caspase 3 is considered to be an authentic molecular biomarker for apoptosis. Caspase 3 is the first activated cysteine-dependent aspartate-specific protease during intracellular apoptosis. It is important in both extrinsic and intrinsic apoptotic pathway. Asp-Glu-Val-Asp-containing peptide substrates at C terminus are recognized and cleaved by activated Caspase 3[153]. Caspase 3 dysregulation (excessive or insufficient) accounts to various number of human diseases like neurodegenerative disorder and cancer. The method for measuring Caspase 3 is more time-consuming and a complex process. Caspase 3 and Caspase 7 are group of Caspase 3 like protease that play a major role in apoptosis and cleave various substrate protein. Caspase 3 is generally present as inactive zymogen but is activated by the caspase-mediated cleavage activator[154]. The mechanism behind this assay is that substrates are cleaved by activated caspases present in the apoptotic process (**Figure 18**). As the caspases are intracellular proteases, protein content for the cell lysate must be prepared first and then the chromogenic or fluorogenic sequence to be cleaved is examined[155]. Caspase 3

expression assay was used by Justin et al.[156] for evaluating apoptosis by curcumin loaded coreshell super paramagnetic iron oxide nanoparticles on HeLa cells.

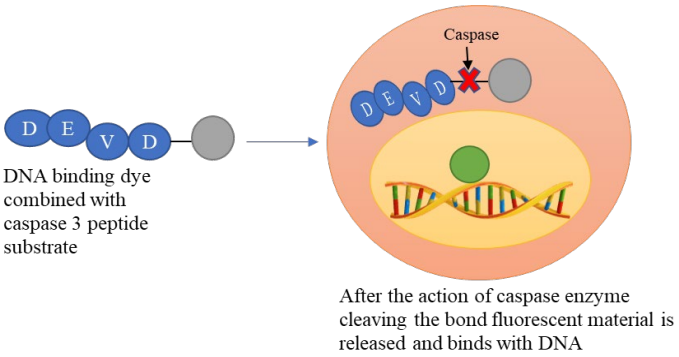

Figure 18. Caspase assay

4. Conclusion

In this review, *in vitro* cell-based assays including dye exclusion assays, colorimetric assays, fluorometric assays, luminometric assays, DNA based assays and apoptotic assays have been discussed. These assays are designed to overcome the ethical issues and challangesfaced while using the animal model. Cell viability can be determined by various new technologies like standard plate readers, etc. Cell based assays must be reliable, rapid, safe, time and cost effective. Each and every assay has its own lead and drawbacks. More literature studies are required regarding the sensitivity of the assays. The nature of the test compound selected must be considered while performing the assay as it may also interfere in the performance of the assay. A single assay cannot be used for examining the cell so it is always recommended to perform more than one assay for confirming the observations..

Ethics statements

The Authors followed MethodsX ethical guidelines, this work does not involve human subjects, animal experiments or data collected from social media.

Funding

This research received no funding

CRediT authorship contribution statement

Antony V. Samrot: Conceptualization, Methodology, Supervision, Writing –review & editing.Subramanian Saigeetha: Methodology, Writing – review & editing; Rajan Renuka Remya: Methodology, Writing –review & editing, **Sivarao Subramonian:** review & editing.

Conflict of interest

The authors declare no conflict of interest

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