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REVIEW ARTICLE

Cell Proliferation and Cytotoxicity Assays

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Abstract: Cell viability is defined as the number of healthy cells in a sample and proliferation of cells is a vital indicator for understanding the mechanisms in action of certain genes, proteins and pathways involved cell survival or death after exposing to toxic agents. Generally, methods used to determine viability are also common for the detection of cell proliferation. Cell cytotoxicity and proliferation assays are generally used for drug screening to detect whether the test molecules have effects on cell proliferation or display direct cytotoxic effects. Regardless of the type of cell-based assay being used, it is important to know how many viable cells are remaining at the end of the experiment. There are a variety of assay methods based on various cell functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity. These methods could be basically classified into different categories: (I) dye exclusion methods such as trypan blue dye exclusion assay, (II) methods based on metabolic activity, (III) ATP assay, (IV) sulforhodamine B assay, (V) protease viability marker assay, (VI) clonogenic cell survival assay, (VII) DNA synthesis cell proliferation assays and (V) raman micro-spectroscopy. In order to choose the optimal viability assay, the cell type, applied culture conditions, and the specific questions being asked should be considered in detail. This particular review aims to provide an overview of common cell proliferation and cytotoxicity assays together with their own advantages and disadvantages, their methodologies, comparisons and intended purposes.

Keywords: Alamar blue, cytotoxicity, Ki-67, raman-microspectroscopy, tetrazolium, viability.

1. INTRODUCTION

Cell viability is the number of living cells in a certain population and measuring the amount of proliferating cells is used as a vital indicator for action of the cell survival or death in response to drugs or chemical agents. The effects of a drug can come up as either cytotoxic, which refers to being toxic and killing the cells or cytostatic, that is defined as inhibition of cell growth. For different types of biological studies, it is important to detect viable cell number in a certain population. Cell cytotoxicity and proliferation assays are basically used for screening the response of the cells against a drug or any chemical agent. Especially pharmaceutical industry widely use cytotoxicity assays to determine the effects of developed agents on the cells. Researchers use different types of assays in order to screen the outcome of a developed therapeutics that target mostly cancer cells.

There are a variety of methods for detecting cytotoxic and cytostatic effects of different compounds and measuring cellular viability. Different methods could rely on various cellular functions including, cell membrane permeability, dye uptake, metabolic activities, enzyme releasing, cell adherence, ATP production, co-enzyme production, DNA synthesis and nucleotide uptake activity. The major and common cell proliferation and cytotoxicity detection methods are classified into different categories.

Trypan blue, a dye exclusion method, is easier to use than all the others, and basically based on staining dead cells over living cells. There are different types of metabolic cell proliferation assays such as MTT, XTT, MTS, WST1, Alamar Blue, calcein AM, LDH release assay and G6PD release assay, which are widely used for screening the cytotoxic effects of drugs. On the other hand, the methods based on DNA synthesis or cell proliferation markers such as H3 thymidine uptake, BrdU, PCNA, phosphohistone H3 or Ki-67 detection are extensively used. In addition to all these assays, ATP assay, protease viability marker assay, clonogenic cell survival assay, sulforhodamine B assays and raman micro-spectrometry are the other common and efficient

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methods for detecting cell viability. However, all these assays should be considered according to the purpose of the study, conditions or cell types. All these types of assays are detailed in below together with their own advantages and disadvantages, their methodologies, comparisons and intended purposes (Table **1**).

2. TRYPHAN BLUE

Dye exclusion methods are based on the usage of different dyes that living cells can exclude but dead cells could not, since living cells with intact cell membrane are highly selective to the passage of molecules through their membrane [1]. Tryphan blue is a negatively charged, ~960 Daltons diazo-dye, which can enter into only damaged membranes and absorbed by dead cells. This dye can be used in either *in vitro*, such as cell cultures or *in vivo*, such as observing cancer cell populations in a living organism [2]. It is also useful for comparing number of viable cells or proliferation rates under certain conditions, between two or more groups. Tryphan blue is frequently used in the laboratories for cell counting under the microscope based on the fact that dead cells turn blue after dye application and living cells are not be stained, having a clear appearance. Although there are many advantages including cheap and easy application, apoptosis or necrosis can not be distinguished by this method and it has very limited sensitivity as compared to other developed assays [2].

3. METABOLIC CELL PROLIFERATION ASSAYS

3.1. MTT Assay

MTT assay is based on the conversion of MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) to insoluble formazan crystals by mitochondrial NAD(p)H-dependent oxidoreductase enzymes released in living cells (Fig. **1**). These mitochondrial enzymes have capability to convert MTT into insoluble formazans by reduction reaction under certain cell culture conditions. MTT assay basically leads us to determine the number of viable cells by measuring mitochondrial activity, which is related with the amount of formazan crystals [3]. Proliferating cells possess higher rate of MTT converting reaction while dead or slow growing cells have low metabolism resulting in lower levels of MTT reduction. After MTT application, formazan crystals are solubilized with a solution such as dimethyl sulfoxide or detergent sodium dodecyl sulfate. Therefore, formazan concentrations can be measured by a spectrophotometer at between 540 to 720 nm [3, 4].

The most common use for MTT assay is to detect cytotoxic effects of different agents under different conditions or different concentrations. It is also possible to detect IC_{50} value (50% half maximal inhibitory concentration) of applied drugs by comparing the viability of control group and drug applied group [5]. MTT assay is suitable for *in vitro* trials of potential drugs and also to study drug resistance in the cell lines. It also helps to determine drug effects *in vitro* and prediction for clinical applications. On the other hand, it is not convenient for follow-up studies since it needs to kill all the cells during protocol. It is also not possible to distinguish cytotoxic and cytostatic agents by this method and results would not be proper when the cell number is low [5, 6].

3.2. XTT Assay

As MTT, XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazo-lium hydroxide) is also a tetrazolium salt, which represents a colorimetric assay to study cellular viability. This method is also based on the reduction of XTT by mitochondrial NADH enzymes converting XTT to formazan crystals [7, 8]. XTT assay causes the generation of a water-soluble type of formazan crystals that distinguishes the XTT assay from MTT assay. The formazan crystals can be solubilized directly in culture medium, thus the additional solubilization step of the procedure would be removed [8]. XTT is easier to use than MTT assay and it is also more suitable for fungal studies. Also, it was suggested that XTT is more sensitive than MTT, and has a larger usage range as compared to other tetrazolium based assays [7].

3.3. MTS Assay

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is another tetrazolium salt, described after MTT and XTT compounds. MTS, in the presence of phenazine methosulphate (PMS), an electron-coupling agent, is reduced to formazan crystals by mitochondrial reductase enzymes in living cells. The reduced formazan crystals are water-soluble, which are dissolved in cell culture medium directly and eliminate the usage of any extra solution or washing step [7]. Formazan crystals can be measured spectrophotometrically at 490-500 nm. MTS reagent solution has more storage stability comparing to MTT or XTT compounds although it needs confirmation with qualified microscope observation. This assay is useful for detecting cell viability in response to drugs, various types of cytokines, growth factors, cytotoxic drugs and anticancer agents [7, 9].

3.4. Water Soluble Tetrazolium Assay

Water Soluble Tetrazolium (WST) salts have been developed as a new generation compounds for cell viability assays that produce formazan crystals by mitochondrial NADH enzymes in living cells. WST-1 and WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) contain an iodine residue and stay more stable in the presence of PMS comparing to XTT or MTS [10]. The WST compounds can also be reduced outside the cell when they are combined with PMS electron mediator and give more efficient spectrophotometric signal than MTT and XTT. WST assays do not need any additional step to wash or solubilize the formazan crystals, and it is marketed as ready-to-use solution making the whole protocol more rapid [10, 11].

3.5. Alamar Blue

Alamar Blue also known as resazurin is a non-toxic cell permeable vital dye. Upon entering into the cell, it is reduced to resorufin, which is red in color and highly fluorescent since they maintain a reducing environment within the cytosol. Higher reduction rate of resazurin to resorufin in proliferating

Table 1. The cytotoxicity/proliferation assays mentioned in this review together with their working principle, advantages and disadvantages.

Method	Principle of Action	Advantages	Disadvantages
Tryphan Blue	Membrane integrity	Widely used Inexpensive Easy to apply	Apoptosis or necrosis can not be distinguished Limited sensitivity
MTT **XTT** **MTS** **WST**	Mitochondrial activity	Widely used Inexpensive Proper for potential drug follow-up studies	Long time incubation Steps in protocol (MTT) Toxic to cell Not suitable for long-term studies Limited sensitivity Work with high cell numbers Can not differ cytotoxic or cytostatic
Alamar Blue	Cell metabolism	Lower toxicity Inexpensive High sensitivity Suitable for long-term studies Highly stable in the culture medium	Fluorescence interference Need long optimization procedure Can not differ cytotoxic or cytostatic
Calcein AM	Cell metabolism	Non-radioactive Rapid process Proper for high-throughput studies	Not very sensitive in adherent cells Need higher sensitive microscopy
LDH Release Assay	Cell membrane integrity & Cell metabolism	Widely used an Accepted Absorbane or fluorescent options	Low sensitivity Not suitable for long-term studies False positive results
G6PD Release Assay	Cell membrane integrity & Cell metabolism	More sensitive than LDH More efficient than LDH Short term protocol	Time-consuming Not suitable for long-term studies
ATP Assay	ATP production	Short term protocol Very good sensitivity Large usage areas	Can not differ cytotoxic or cytostatic
Sulforhodamine B Assay	Cellular protein content	Suitable for high-throughput screening Proper for long-term studies High sensitive in low cell numbers	Can not differ living or dead cells
Protease Viability Marker Assay	Differential protease activity	Excellent correlation with established methods Combatible with downstream applications Non-toxic Short incubation time Differ living or dead cells	No significant advantages
Clonogenic Cell Survival Assay	Proliferation ability of the cell	Suitable for long-term cytotoxicity screening Consider reversible damage or resistance against agents	Limited dynamic range Not suitable to test the impacts of agents that do not target DNA synthesis
H^3 Thymidine Uptake	DNA replication	No significant advantages	Harmful effects Difficulties in handling Time-consuming
BrdU	DNA replication	Reduced equipment and time Suitable with simultaneous techniques	Harsh labelling conditions Potential carcinogen
Ki-67 Antigen	DNA replication and cell division	All phases of the cell cycle and mitosis detected	Not suitable for formalin-fixed paraffin sections
PCNA Detection	DNA replication	No detrimental effect on histological architecture	Variations in signal intensities PCNA's longer half life
Phospho-Histone H3 Detection	Cell division	All four stages of mitosis detected	Time-consuming
Raman Micro-Spectrometry	Interaction of electromagnetic radiation with the sample molecule	Very rapid Non-invasive No damage to the cells	No significant advantages

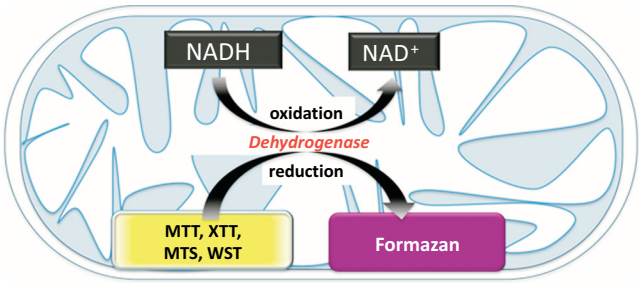

Fig. (1). MTT assay. Mitochondrial NADH is converted to NAD+ via dehydrogenase. This reaction causes the reduction of yellow tetrazolium salts (MTT, XTT, MTS and WST) to purple formazan crystals in living cells.

cells and lower rate of this reduction in dead cells could be a marker to distinguish cellular viability. Dead or damaged cells would create lower fluorescence changes since they are lack of cellular metabolism [12]. The metabolic reactions generating fluorescence signals can be measured at 570 and 630 nm.

This method can be preferred since it causes lower toxicity than the other methods and most importantly this dye can be used to conduct long-term experiments without killing the cells [13]. Also, resazurin is a non-radioactive, water-soluble molecule that has high stability in the cell culture. On the other hand, using resazurin may require very long experimental optimization time that decreases its convenience for rapid assays. In some cases, it causes fluorescence interferences, which may cause false results [14].

3.6. Calcein-AM

Although calcein is a type of fluorescent dye, being detected at 495/515 nm wavelength, its non-fluorescent derivative calcein-acetomethoxy (Calcein AM) is commonly used in cytotoxicity assays [15]. Calcein AM can pass the membrane in both living and damaged or dead cells with the help of its lipid soluble feature. After the transport of calcein AM into the cells, cytoplasmic esterases remove its acetomethoxy group and calcein give a strong green fluorescent signal [15, 16]. Cytoplasmic esterases can not be activated in dead or damaged cells, and thus only living cells are stained with calcein and the signal they give out can be measured rapidly in a sensitive manner by flow cytometry. Calcein assay is useful for clinical laboratory procedures and it is described as a quantitative, standardized, easy to use and inexpensive method. Up to now, it was shown that calcein AM assay can be used in drug resistance studies such as P-glycoprotein and multidrug resistance protein activities, drug-drug interactions as well as red blood cell and aging studies. On the other hand, it is known that this assay is not proper for adherent cells because the adherence can block the passage of calcein into the cells [16, 17].

3.7. Lactate Dehydrogenase Release Assay

Lactate dehydrogenase release assay was first developed to determine cytotoxicity in immune cells. LDH is a soluble cytoplasmic enzyme released into the cell culture medium after membrane disruption in necrotic or apoptotic cells [18]. Therefore, LDH activity can be measured to detect the cytotoxic effects of different agents or environmental factors. LDH assay includes 2-step procedure to measure the level of LDH activity in cell culture medium. In the first step, LDH catalyzes the conversion of lactate to pyruvate via reduction of NAD+ to NADH. Then, this step is followed by the reduction of a tetrazolium salt (INT) to a red formazan product by diasphorase enzymes using generated NADH [4, 19] (Fig. **2**). Lastly, appeared tetrazolium salt can be measured colorimetrically at 490-520 nm by a spectrophotometer. Therefore, the level of formazan formation is directly proportional to the amount of released LDH in the medium. The LDH level in the culture medium can be contaminated by endogenous LDH activity and cause false positive results, suggesting additional confirmations are needed with this method. Although this method is used widely and accepted, LDH half-life in the culture medium is limited and the sensitivity of LDH assay is also limited [19, 20].

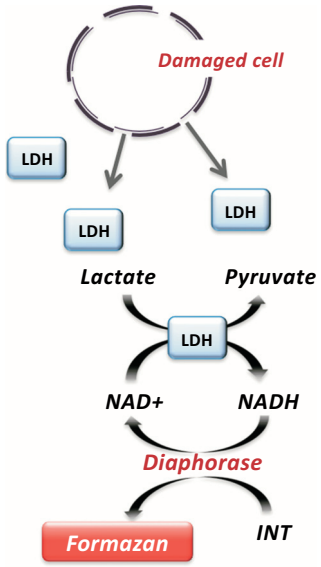

Fig. (2). LDH release assay. LDH released from damaged or dead cells catalyzes the oxidation of lactate to pyruvate while catalyzing NAD+ reduction to NADH molecule. The resulting NADH is used by Diaphorase enzyme to transform INT (tetrazolium salt) to red formazan crystals, which is measured colorimetrically.

3.8. Glucose-6-phosphate Dehydrogenase Release Assay

Glucose-6-phosphate dehydrogenase (G6PD) assay is also a fluorescence based cell viability assay. This assay is based on the releasing of cytosolic enzyme, G6PD, from damaged cell membrane to the culture medium in dead cells. G6PD is involved in pentose phosphate pathway and responsible for generating NADPH from $NADP^+$ in the mitochondria. Exogenously added resazurin can be reduced to resorufin, which is a highly fluorescent dye, in the presence of generated NADPH. G6PD assay is based on measuring the amount of fluorescent signal to determine the concentration of dead cells in the population. G6PD assay is more sensitive comparing to LDH. Moreover, it was shown that background signals in G6PD assay are lower than LDH assay, which confirm the efficiency of this assay. On the other hand,

G6PD release assay can be completed in a very short time as compared to other enzyme release assays [14].

4. ADENOSINE 5'-TRIPHOSPHATE ASSAY

Adenosine 5'-triphosphate (ATP) is the major energy source of living organisms and mostly produced in mitochondria. ATP and its phosphate bond supply the energy to complete all the energy requiring processes in the cells. Measurement of total cellular ATP levels can lead to determining cell viability or cytotoxic effects of different agents on the cells [21].

ATP assay is based on monitoring of ATP levels via bioluminescent detection. Added natural firefly enzyme luciferase and luciferin react with cellular ATP and cause the production of certain level of light that is measured by a Luminometer (Fig. **3**). The amount of luminescent light is directly proportional to the viability of cells [21, 22]. ATP assay is advantageous comparing to other methods with different ranges such as short time protocol, high sensitivity and large usage areas. The signal is also present for a long time of period that makes the measuring step easier. But it is not possible to differentiate cytostatic or cytotoxic effects of drugs since the decrease in ATP level could depend on cell number, cell death or metabolic activities [4]. On the other hand, it was shown that ATP assay is more accurate than all other assays in terms of predicting the resistance or sensitivity of platinium-resistant epithelial ovarian cancer tumors to other agents [23].

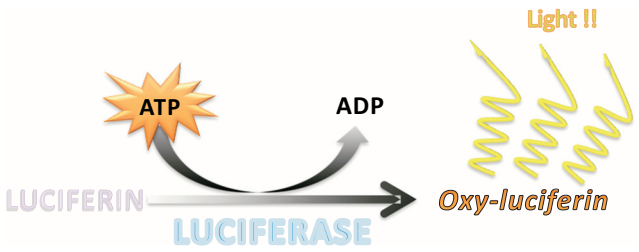

Fig. (3). The principle of ATP assay. Exogenously added luciferin is turned into oxy-luciferin by luciferase enzyme with a reaction involving cellular ATP. The oxy-luciferin producing strong fluorescent light is captured and measured by a Luminometer.

5. SULFORHODAMINE B ASSAY

Sulforhodamine B (SRB) (C27H30N2O7S2) assay was developed by Skehan *et al*. for measurement of *in vitro* cytotoxicity, proliferation and drug screening. SRB assay is based on the binding of SRB dye to all protein components via their basic amino acid residues of trichloroacetic acid (TCA) fixed cells under mild acidic conditions. SRB dye is an anionic bright-pink aminoxanthene and has two sulfonic groups that are capable of binding basic amino acids electrostatically and pH dependently [22, 24]. Then, associated SRB dye can be solubilized from the cells using weak bases and measured spectrophotometrically at 490-550nm. The amount of SRB concentration is directly proportional to the number of living cells.

It was shown that the SRB assay is highly sensitive even to detect the optic density (OD) value of 1,000-2,000 cells [22]. Moreover, it is known that SRB assay is more practical than the other methods because the cells can be stored as long as required after TCA fixation and SRB staining. SRB assay exhibits the same performance level comparing to MTT or clonogenic assays when the test is limited to IC_{50} value of the agent. On the other hand, MTT requires cellular activity to exhibit the results and it detects the living cells while SRB can not differentiate living or dead cells but just represent the data of total protein amount which can be used as a factor to measure living cells. Once the protocol has been optimized, the SRB assay can used for high-throughput screening applications by the help of automated equipments [24, 25].

6. PROTEASE VIABILITY MARKER ASSAY

Mammalian cells in the culture have a great diversity in terms of enzymes such as proteases which are involved in several cellular processes like intracellular protein degradation and cell division and grouped into cytoplasmic, lysosomal and transmebrane-bound proteases. The activities of proteases are regulated by distinct mechanisms such as unique compartmentalization [26]. Therefore, a protease viability assay is developed based on the detection of differential ubiquitous proteolytic activities associated with intact viable cells and cells that have lost membrane integrity. The release of stable protease activity into the medium and the measure of constitutive proteolytic enzymes could allow simultaneous measurement of the relative number of viable or dead cells in a cell culture population [27].

Recently, the measurement of a conserved and constitutive protease activity within live cells has been developed as a marker of cell viability. A fluorogenic cell permeable protease substrate (glycyl-phenylalanyl-aminofluorocoumarin; GF-AFC) has been used to selectively detect protease activity in viable cells. The glycine and phenylalanine amino acids are removed from the GF-AFC by cytoplasmic aminopeptidase activity to release aminofluorocoumarin (AFC) when the GF-AFC substrate penetrates into live cells, and thus generating a fluorescent signal proportional to the number of viable cells [27]. The protease responsible for the cleavage of the GF-AFC substrate becomes inactive as soon as the cells die. On the other hand, a second protease is released into the medium from dead cells since they have lost membrane integrity. This protease activity is measured using a nonpermeable fluorogenic peptide substrate (bis-alanylalanyl-phenylalanyl-rhodamine 110; bis-AAF-R110). Because the AAF-R110 substrate is nonpermeable and cannot enter to viable cells, the signal from this substrate is selective for the dead cell population [27, 28].

In methodology, the GF-AFC and AAF-R110 substrates can be added together as a single reagent to cells in culture and incubated for 30 minutes to generate fluorescent signals that are proportional to the number of viable and dead cells [28].

This viability assay has important advantages: I) The viable and dead cell protease markers show excellent correlation with established methods such as measurement of ATP and release of lactate dehydrogenase, II) The GF-AFC and AAF-R110 substrates are compatible with other reagents involved in additional downstream applications to measure

other parameters, such as gene expression or caspase activity, III) The GF-AFC substrate is relatively non-toxic to cells in the culture even when the cells are exposed to the GF-AFC for longer times and longer exposure results in only very small changes in the viability, IV) The incubation time required to measure a signal is much shorter (about 30 min to 1 hour) than the tetrazolium assays (1 to 4 hours) [29, 30].

7. CLONOGENIC CELL SURVIVAL ASSAY

The clonogenic cell survival assay or colony formation assay is based on the ability of a cell to proliferate to form a large colony or a clone. This cell is then said to be clonogenic. A cell survival curve is designed based on a relationship between the dose of the agent used and the proportion of cells retaining their ability to reproduce [31]. Although this method is used as a first choice to study the effects of radiation on cells, it is now commonly used to determine the effects of other cytotoxic agents [32]. To study the effects of various agents alone or in combination, most laboratories commonly use established cell lines for clonogenic cell survival assay.

In methodology, cell suspension is prepared by deattaching the cells from an actively growing stock culture in monolayer and the number of cells per milliliter in this suspension is then counted using a hemocytometer. Before or after treatment, the appropriate number of the cells are seeded into a dish and incubated to form colonies in 1 to 3 weeks. Each single cell divides many times and forms a colony. Colonies are fixed with glutaraldehyde, stained with crystal violet, and counted using a stereomicroscope. Then, the plating efficiency (PE), the percentage of cells seeded into a dish that finally grow to form a colony, is calculated for both treated and untreated control cells [31, 32].

Various non-clonogenic cytotoxicity and cell survival assay can be used to measure the potency of a cytotoxic agent, however these short-term assays can underestimate cytotoxicity as compared to long-term cell growth assays. On the other hand, non-clonogenic cytotoxicity assays can sometimes overestimate cytotoxicity due to not considering reversible damage or regrowing of resistant cells against the cytotoxic agent. For these reasons, it may be suggested to use clonogenic cell survival assays [4].

The clonogenic cell survival has some limitations as well: I) It lacks dynamic range (the ratio between the largest and smallest possible values of a changeable quantity) of fluorescent methods or the ATP assay [4, 33], II) It can not measure the effects of cell to cell communication on cell proliferation due to low density of the cells plated, III) This assay is not suitable to test the impacts of agents that decrease growth without inhibiting DNA synthesis and/or cell cycle progression and the agents that inhibit growth only by causing cytoskeletal damage or by inducing apoptosis [34, 35].

8. METHODS FOR DETECTING DNA SYNTHESIS AND CELL PROLIFERATION MARKERS

Cellular division and DNA replication are fundamental biological activities, which play important roles in both physiological and pathological processes. It is important to know when S phase (DNA synthesis) occurs precisely in the cell cycle in order to maximize the tumor killing effects of anticancer drugs targeting cell cycle [36]. Therefore, the data obtained from cell kinetic data gives valuable information after treatment, especially in many cancer types. In this context, the use of monoclonal antibodies against specific cell proliferation-associated antigens is the most common approach. These antibodies could detect either endogenous molecules or exogenous substances which can be taken up by cells at specific stages during the cell cycle [37].

8.1. Tritium Labeled (H^3) Thymidine Uptake

The original approach to detect mitotically active cells included the incorporation and detection of tritium labeled (H^3) thymidine using autoradiography or scintillation techniques. Tritium labeled (H^3) thymidine and autoradiography were originally developed as DNA probes to prove that DNA replication is a semi-conservative manner [36]. This method has harmful effects, including DNA fragmentation, cell cycle arrest, chromosomal aberrations, and induction of apoptosis [38]. Moreover, difficulties in handling radiolabeled substrate, the time-consuming nature of this technique and the detection of the cells only in the S phase resulted in the development of new techniques to label nascent DNA, which was facilitated by advances in the production of monoclonal antibodies [39].

8.2. 5-bromo-2-deoxyuridine (bromodeoxyuridine or BrdU)

DNA synthesis can be detected by measuring the incorporation of the thymidine analogue, bromodeoxyuridine (BrdU), in place of thymidine during the S phase of the cell cycle. Therefore, this nucleoside analog becomes an excellent marker of cell cycle and proliferation. The development of a specific antibody against BrdU provided an opportunity for immunological detection of the newly synthesized BrdU-incorporating DNA.

BrdU method has numerous advantages over tritium labeling such as significantly reduced equipment and time. This method enables the use of several simultaneous techniques and processes such as cell fate and lineage analysis using cell specific markers as well [39]. As well as its advantages, there are a number of limitations in its utility. For instance, the detection of BrdU following its incorporation into DNA needs the denaturation of DNA to allow BrdU specific antibody binding and the cell and nuclear membranes have to be permeabilized for antibody access. However, these harsh labeling conditions could cause cell disruption together with protein and nucleic acid degradation, which limit the use of this method if simultaneous measurement of protein content or molecular analysis is required. [40]. Moreover, researchers working with BrdU should be careful because it is classified as a potential carcinogen. Despite these negative outcomes, BrdU has remained the first choice to study cell proliferation.

At present, there are several ways suitable for both adherent and suspension cells that allow the quantification of BrdU incorporation into the newly synthesized DNA of actively proliferating cells such as colorimetric and chemiluminescent options based on the method of choice [41, 42].

As well as these approaches, BrdU incorporation may be quantitated by flow cytometry or immunohistochemistry. However, the use of flow cytometry is limited to cell suspensions, thus it is not suitable to detect the localization of specific S phase cells in tissue samples. Immunohistochemical techniques-related detection of BrdU incorporation is appropriate for S phase cells [39, 40].

8.3. Detection of Ki-67 Antigen

Ki-67 is a nuclear antigen associated with the cell cycle. A detailed cell cycle analysis displayed that the antigen was expressed in all phases of the cell cycle (G1, S, and G2 phases) and mitosis. However, quiescent or resting cells in the G0 phase did not express Ki-67 antigen [43]. Although the exact function of Ki-67 antigen during the cell cycle is unknown, there is a close link between its protein expression and proliferation. Because, the Ki-67 antigen is found in all proliferating cells including normal and cancer cells, the presence of this structure is an excellent marker to determine the fraction of growing cells in a population. Therefore, antibodies against the Ki-67 protein are widely used in cancer research [44]. The percentage of cells staining positive for the Ki-67 antigen is called the Ki-67 index and it is directly related to cell proliferation. The Ki-67 index may give an idea about the growth characteristics of a tumor and the effects of certain drugs on it.

The Ki-67 index is more sensitive as compared to the determination of the cellular DNA content by flow cytometry and *in vitro* labeling of newly synthesized DNA since the Ki-67 antibody recognizes cells in all active phases of the cell cycle [44].

The Ki-67 staining protocols show variation based on its detection method. It could be detected using flow cytometry-related approach or colorimetric approach. In both methods, the cells are fixed and become permeabilized. Then, anti-Ki-67 antibody is added to the cells. Based on the feature of Ki-67 antibody, a secondary antibody recognizing the Ki-67 antibody is added and then analysis is performed [45,46]. Additionally, immunostaining of Ki-67 antigen on various types of cytological and histological preparations provides a great opportunity to count immune histologically stained nuclei. However, the original human Ki-67 antibodies are not suitable for formalin-fixed paraffin sections that are commonly used in histopathology and these antibodies have limited cross-species reactivity. However, these drawbacks have been solved by producing new monoclonal antibodies named as MIB (for Molecular Immunology Borstel), which can detect Ki-67 equivalents in several different species such as cattle, dog, horse, sheep and rodents and can be used with formalin-fixed, paraffin-embedded tissue [44].

8.4. Proliferating Cell Nuclear Antigen Detection

Proliferating cell nuclear antigen (PCNA) is an evolutionarily well-conserved 36-kDa protein, which was firstly identified as a processivity factor of DNA polymerase δ required for DNA synthesis during replication. PCNA provides processivity through association with various DNA replication associated proteins. On the other hand, PCNA is involved in many other important cellular processes such as DNA repair, chromotain remodeling and cell cycle control [47]. It is known that PCNA synthesis begins in the late G1 phase, makes a peak during the S phase, declines in G2 and is virtually absent in the M phase [48]. Therefore, PCNA is often used as a marker for cell proliferation due to its vital roles in DNA replication.

Several monoclonal antibodies against PCNA have been developed such as 19A2 and PC10 that are commercially available. These antibodies are used to detect PCNA based on their specific features by using various techniques such as immunofluoresence staining of fixed cells, immunohistochemical analysis of formalin-fixed, paraffin-embedded tumor tissues and flow cytometry [49]. At present, there are also commercial PCNA elisa kits for nuclear and whole cell extracts.

A major advantage of using PCNA antibodies is that they can be applied to fixed tissue without any detrimental effect on histological architecture [50]. The major limitations of this technique are varied staining intensities of nuclei, dependence of staining on fixation method (methanol fixation results in the labeling of cells in S phase, whereas other types of fixation permit the staining of all cycling cells) and PCNA's longer half life (20 hour, it can be expressed even when the cells reach G0) are [51].

8.5. Phospho-Histone H3 Detection

Phospho-Histone H3 (pHH3) is a recently described proliferation marker and it is specific for all four stages of mitosis [52]. The phosphorylation of histone H3 plays an important role in gene expression, chromatin remodeling, chromosome condensation and cell division. It is known that histone H3 phosphorylation may occur at different phases of cell division depending on different organisms. However, metaphase chromosomes are always found to be heavily phosphorylated [53]. H3 phosphorylation at serine 10 and serine 28 is coupled with mitotic chromosome segregation and condensation [54]. Development of anti-pHH3 antibody makes the immunohistochemical detection easier in cells and tissues. Therefore, pHH3 staining may be a useful method in which accurate determination of proliferation potential is relevant to tumor grading or clinical treatment decision-making [54].

In a current protocol, antigen detection in tissues and cells is a multi-step immunohistochemical process. The initial step involves the binding of the primary antibody to its specific epitope. After this labeling, an enzyme labeled polymer is added to bind to the primary antibody. The detection of the bound antibody is performed by a colorimetric reaction [55].

9. RAMAN MICRO-SPECTROSCOPY

Most of the techniques described in the literature are invasive and require additional steps such as cell fixation, staining or protein extractions. Raman microscopy is used to distinguish cells at different stages of cell cycle and living cells from dead cells as a non-invasive method. The working principle of raman spectroscopy includes the interaction of electromagnetic radiation with the sample molecules and represents a chemical fingerprint of the sample [56]. This technique has three main advantages over conventional

biological assays when applied to the study of living cells: I; it is very rapid (1 to 3 min), II) no labels are required so it is non-invasive, and III) no damage is induced to the cells if suitable laser wavelength and intensities are used [57]. Raman spectroscopy has been shown to analyze *in situ* and real time biochemical changes in living cancer cells treated with toxic chemical agents [58]. In a recent study, the effect of cisplatin on A549 human lung adenocarcinoma cells was investigated with raman spectroscopy. The results were compatible with MTT cytotoxicity assay [59].

10. CONCLUSION

The measurement of cell viability has a significant and fundamental role in all cell culture studies. Cell cytotoxicity and proliferation assays are generally used for drug screening to detect whether the test molecules have effects on cell proliferation or display direct cytotoxic effects. Regardless of the type of cell-based assay being used, it is important to know how many viable cells are remaining at the end of the experiment. There are wide arrays of cell viability methods ranging from the most routine tryphan blue dye exclusion assay to highly complex analysis of individual cells, such as using Raman microscopy. In order to choose the optimal viability assay, the cost, speed, and complexity of equipment, the cell type, culture conditions, the specific questions being asked should be considered in detail. Regardless of the assay method, there are major critical factors that should be considered for reproducibility and success: I) experiments should be performed with tightly controlled and consistent source of cells and II) drug/agent/reagent concentration and incubation time should be optimized for each experiment.

CONFLICT OF INTEREST

The authors do not have any kind of conflict of interest affecting the compilation of the current knowledge in this area for writing this review.

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