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Abstract

Apoptosis has been recognized as a type of programmed cell death connected with characteristic morphological and biochemical changes in cells. This programmed cell death plays an important role in the genesis of a number of physiological and pathological processes. Thus, it can be very important to detect the signs of apoptosis in a study of cellular metabolism. The present paper provides an overview of methods often being used for detecting DNA fragmentation as one of the most specific findings in apoptosis. To date, three routine assays have been developed for detecting DNA fragmentation: DNA ladder assay, TUNEL assay, and comet assay. All these methods differ in their principles for detecting DNA fragmentation. DNA ladder assay detects the characteristic “DNA ladder pattern” formed during

	internucleosomal cleavage of DNA. Terminal deoxynUcleotidyl transferase Nick-End Labeling (TUNEL) assay detects DNA strand breaks using terminal deoxynucleotidyl transferase catalyzing attachment of modified deoxynucleotides on the DNA strand breaks. Comet assay can be used for detecting nucleus breakdown producing single/double-strand DNA breaks. The aim of this review is to describe the present knowledge on these three methods, including optimized approaches, techniques, and limitations.
Keywords (separated by '-')	Apoptosis - DNA fragmentation - Apoptosis assays - DNA ladder - TUNEL assay - Comet assay
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REVIEW

An overview of apoptosis assays detecting DNA fragmentation

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Abstract

Apoptosis has been recognized as a type of programmed cell death connected with characteristic morphological and biochemical changes in cells. This programmed cell death plays an important role in the genesis of a number of physiological and pathological processes. Thus, it can be very important to detect the signs of apoptosis in a study of cellular metabolism. The present paper provides an overview of methods often being used for detecting DNA fragmentation as one of the most specific findings in apoptosis. To date, three routine assays have been developed for detecting DNA fragmentation: DNA ladder assay, TUNEL assay, and comet assay. All these methods differ in their principles for detecting DNA fragmentation. DNA ladder assay detects the characteristic "DNA ladder pattern" formed during internucleosomal cleavage of DNA. Terminal deoxynUcleotidyl transferase Nick-End Labeling (TUNEL) assay detects DNA strand breaks using terminal deoxynucleotidyl transferase catalyzing attachment of modified deoxynucleotides on the DNA strand breaks. Comet assay can be used for detecting nucleus breakdown producing single/double-strand DNA breaks. The aim of this review is to describe the present knowledge on these three methods, including optimized approaches, techniques, and limitations.

Keywords Apoptosis · DNA fragmentation · Apoptosis assays · DNA ladder · TUNEL assay · Comet assay

Introduction

Apoptosis, which term was first used by Kerr, Wyllie and Currie in a paper from 1972, is a complex process responsible for removing damaged cells from living organisms [1]. Apoptosis has been characterized as a type of programmed cell death connected with characteristic morphological and biochemical changes of the cells. To date, three main activation pathways for apoptosis have been described. These are termed the extrinsic, intrinsic, and perforin/granzyme-mediated pathways. All these pathways can lead to activation of caspase-3, which mediates cell death through additional cell damage [2, 3].

A number of different proteins participate in the apoptotic cascade. These are detectable using common analytical methods based on protein detection. The rate and stage of apoptosis is frequently characterized using detection of the activity of caspases, which are enzymes (i.e., proteases) specifically cleaving peptide bonds of appropriate substrate. To date, 11 isoenzymes of caspases have been described in human whereas seven of them (i.e., caspase 2, 3, 6, 7, 8, 9, 10) participate in apoptosis [4, 5].

Apoptosis plays a crucial role in the pathogenesis for a number of pathological and physiological processes. Problems also can arise either due to excessive apoptosis [6] or to reduced apoptosis [7]. Thus, it is very necessary to detect the signs of apoptosis in order to improve and expand upon the possibilities for slowing or even obstructing the progress of such diseases. Moreover, detection of apoptosis is an important indicator in testing potential new medicaments and the general cell-toxicity of chemicals.

Detecting morphological changes in apoptotic cells

Apoptosis includes morphological and biochemical changes in the cell, and these can be used for its detection. The morphological changes during apoptosis include shrinkage of the cell, pyknosis (=chromatin condensation), and karyorrhexis

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(nucleus fragmentation) followed by DNA fragmentation. The cytoskeleton of the cell is damaged, thereby allowing membrane blebbing. In the late stage of apoptosis, the apoptotic bodies are formed [8–10].

A number of microscopy techniques have been used for determining morphological changes in the cell [1, 11, 12], including, among others, light microscopy and electron microscopy [1, 12–15]. Transmission electron microscopy can be used for determining ultrastructural changes and chromatin condensation within the cells [15], and scanning electron microscopy is appropriate for detecting cell surface changes [16–18]. Even atomic force microscopy could be used to determine morphological changes of the cells during apoptosis [19, 20]. In addition, phase-contrast microscopy and, most often, fluorescence microscopy can be used [21].

The important biochemical feature of apoptosis is the exposure of phosphatidylserine on the outer part of the plasma membrane of apoptotic cells [22]. This phosphatidylserine exposure serves as a signal for macrophages eliminating the apoptotic cells. Annexin V staining is usually used to detect phosphatidylserine. Annexin V binds to phosphatidylserine in the presence of Ca^{2+} ions. Because annexin V is labeled using fluorescein isothiocyanate (FITC) [23, 24], it allows the detection of phosphatidylserine using fluorescence microscopy. Annexin V staining can be positive during necrotic process [24], thus double staining using annexin V and propidium iodide is essential to confirm apoptosis [25].

Fluorescence microscopy is often utilized also for evaluating cell nucleus damage and DNA fragmentation in apoptosis using nucleus blue acid stains 4′,6′diamidino2phenylindole (DAPI) or HOECHST 33258, 33342 and 34580. DAPI and Hoechst 33258 bind on A-T base pairs in the minor-groove of double-stranded DNA [26, 27]. The main difference between them is that Hoechst 33258 visualizes DNA also in living cells and thus allows analysis of the nucleus in real time [27]. Flow cytometry is another technique to detect apoptosis in cells. Apoptotic cells can be identified as the fractional subG(1) population using propidium iodide [28–30].

Detecting DNA fragmentation in apoptotic cells

DNA fragmentation

DNA fragmentation is the main feature of apoptosis, and thus it is used as a marker of apoptosis. The mechanism of DNA cleavage is illustrated in Fig. 1. Double-stranded DNA is cleaved by DNA fragmentation factor (DFF) [31]. DFF is a heterodimer consisting of 40 kDa catalytic subunit (DFF40) and 45 kDa regulatory subunit (DFF45) [32]. DFF 40 has endonuclease activity at neutral pH in the presence of Mg^{2+} [33] and cleaves double-stranded DNA specifically, with a preference for A/T-rich region [34].

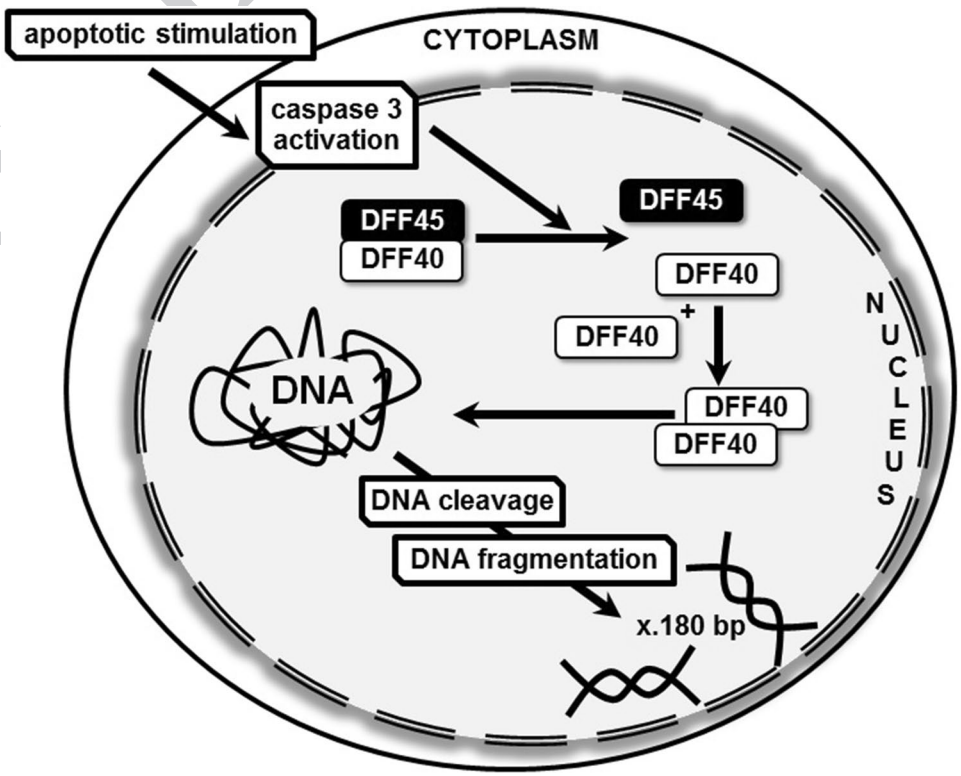

Fig. 1 Mechanism of DNA fragmentation during apoptosis. DNA fragmentation factor catalytic subunit (DFF40) forms a complex with the inhibitor of DFF40 (DFF45). During apoptosis, the DFF40-DFF45 complex is cleaved by activated caspase 3 (a-caspase 3), which is formed by cleavage of pro-caspase 3 through the apoptotic stimuli. DFF40 dimerizes and cleaves DNA in internucleosomal linkers into fragments of 180 bp and multiples thereof

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Under normal conditions, DFF40 is inhibited by the inhibitor DFF45. DFF45 also serves as a chaperon for DFF40 during its synthesis [35]. During apoptosis, procaspase 3 is cleaved to the activated caspase 3, which in turn cleaves the DFF45–DFF40 complex, and thus DFF40 is activated [36]. DFF40 cleaves nuclear DNA into internucleosomal fragments about 180 bp in size and multiples thereof (e.g., 180, 360, 540, 720 bp). This so-called "DNA ladder" pattern has been used for identification of apoptosis in cells since 1976, when Skalka et al. proved the cleavage of chromatin DNA in lymphoid tissues of irradiated mice in vivo [37]. In 1980, Wyllie proved the cleavage of internucleosomal DNA in thymocytes treated by glucocorticoids undergoing apoptosis [13]. Because DNA fragmentation is a specific marker of apoptosis, methods have been developed for using it in detecting and characterizing cellular apoptotic processes. To date, three main methods have been developed for detecting DNA fragmentation: DNA ladder assay, TUNEL assay, and comet assay.

DNA ladder assay

DNA ladder assay uses the presence of the "DNA ladder" pattern of DNA fragments occurring during apoptosis. Key steps in the detection methodology are as follow: First, cultured cells are harvested, cells are lysed, fragmented genomic DNA is isolated, then contaminating RNA is digested. Next, the negatively charged DNA fragments are separated on agarose gel under direct electric current, whereby the DNA migrates to the anode. Finally, the DNA fragments are stained and visualized. A characteristic "DNA ladder" pattern is shown in Fig. 2.

DNA-ladder assay involves culturing the tested cells under defined conditions and with a chemical substance of interest for an appropriate duration. Before cell lysis of adherent cells, it may be necessary to take into account also that some cells had previously detached themselves from the cultivation surface. Thus, the cell medium might be centrifuged to assemble floating apoptotic cells [38].

Lysis buffers of varying composition are used for lysing mammalian cells. Lysis buffers generally contain tris(hydroxymethyl)aminomethane (*Tris*) and ethylenediaminetetraacetic acid (EDTA) [39, 40] with sodium chloride (NaCl) [12, 38, 41] as the main components at pH 7.5. Dimethyl sulfoxide (DMSO) also can be used for cell lysis [42]. Isolation of the fragmented DNA is then done using such common methods for genomic DNA isolation as phenol–chloroform [15, 41, 43, 44], or phenol–chloroform–isoamyl alcohol extraction [45]. A number of isolation procedures based on various physical and chemical principles are used for isolating apoptotic low molecular weight DNA fragments from apoptotic cells [46–52]. Commercial kits for this purpose mostly use solid-phase extraction on, for example, silica gel or glass fiber fleece. Isolation of apoptotic DNA fragments using commercial kits is faster, safer, more sensitive [46], and simpler in comparison to the standard phenol-chloroform extractions. A disadvantage of using commercial kits is their greater cost.

Fig. 2 DNA ladder pattern. *a* Control cells, *b* cells exposed to a apoptotic agent

Usually, purification of fragmented DNA from RNA is included in the DNA ladder assay methodology. The digestion of contaminating RNA is performed using RNase A in different concentrations up to 0.1 mg/mL [15, 43, 53–56].

After DNA isolation and purification, electrophoresis is performed on agarose gel. Agarose gel is used at varying concentrations within the range of 1–2% [15, 38, 45, 48, 50–53, 55–63]. The voltage applied depends upon the size of the DNA fragments to be distinguished, but the usual rate is in the range of 2–15 V/cm [44, 55, 64]. Because smaller DNA fragments are more sensitive to heating, lower voltage should be used where these are involved. The DNA is most often visualized using ethidium bromide that is added to the agarose gel during preparation [12, 15, 44, 45, 48, 50–53, 56–58, 64]. Because ethidium bromide is a strong mutagen, a safer and environmentally friendlier alternative, SYBR-Safe, may be preferred for DNA gel staining [38]. The standard visualization is performed using an ultraviolet transilluminator with excitation/emission wavelengths depending on the fluorescent dye used. The characteristic "DNA ladder" pattern (Fig. 2) can then be observed.

DNA ladder assay is relatively easy to perform and does not require special equipment. It has sensitivity on the order

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of 10^6 cells [39–41, 57], thus making it an inappropriate method for samples having lower numbers of apoptotic cells but very useful for experiments on cell cultures or tissues with high numbers of cells. The crucial limitation of DNA ladder assay for use in apoptosis estimation is that DNA fragments could occur also during necrosis [65, 66], and thus a "smear pattern" could be observed in the case of necrosis [67].

Another drawback is that an absence of DNA ladder pattern does not prove that no apoptotic cells were occurring in a tested sample [2, 68]. This test should be used only for proving apoptosis at a later stage when apoptosis is believed to have been ongoing, because the internucleosomal cleavage of DNA is an event occurring late in the apoptotic process [69]. Thus, it is necessary and very common to confirm the apoptosis in tested cells using another assay based on a different principle.

Comet assay

Comet assay, also known as "single cell gel electrophoresis assay" (SCGE), is a rapid method used for detecting DNA damage or repair in a single cell [70]. It has been increasingly used in genotoxicity testing [71].

The principle underlying comet assay originated in the 1970s [72], but the assay itself was introduced in 1984 by Östlink and Johanson [73]. These authors used comet assay for detecting DNA strand breaks caused by ionizing radiation of mammalian cells, and they improved the sensitivity of the original method by using agarose gel electrophoresis for DNA fragments distribution. After separation of DNA on the agarose gel, the DNA pool has the appearance of a comet, thus giving the assay its name. The assay was first described for apoptosis detection by Olive et al. [74]. Comet assay has been used for detecting both single-strand (ssDNA) breaks under alkaline conditions and double-strand DNA (dsDNA) breaks under neutral conditions [75].

Alkaline conditions at pH 10 [76] or higher [77] enable the detection of single-strand DNA (ssDNA) breaks. Alkaline pH disrupts the nonbinding interactions between nitrogenous bases in DNA so that DNA strains are separated. Thus, ssDNA breaks are released and become detectable. Neutral pH (~7) is appropriate for detecting dsDNA breaks because the separation of DNA strains does not occur under neutral pH [74]. Recently, this separation has been shown to occur also within a combination of denaturing and nondenaturing conditions. This approach enables simultaneous evaluation of ssDNA and dsDNA strand breaks and thus is called 2T-comet assay, short for "two-dimensional perpendicular tail comet assay" [78]. It has been used for detecting DNA strand breaks in human spermatozoa.

The comet assay procedure consists of (1) fixation of the analyzed cells on a microscope slide, (2) cell lysis, and (3) agarose gel electrophoresis in direct electric current. Finally (4), the DNA is stained and visualized. If DNA strand breaks exist, the comet is observed on the gel (Fig. 3).

The detailed experimental procedure consists of the four steps listed above. The estimated cells are mixed with up to 1% low melting point agarose (LMPA) [79–86], which (in contrast to commonly used agarose) is liquid under normal conditions. The mixture of cells and LMPA is placed on a microscope slide covered with normal melting point agarose (NMPA) in concentration ranging between 0.5% and 1% [79–82, 84, 86, 87]. The cover glass is placed over the mixture and agarose and the slide and contents are tempered at 4 °C for 5 min to cause solidification of the LMPA. To lyse cells, the slide is submerged in lysis buffer, which has a composition similar to those of lysis buffers used for DNA ladder assay. Tris (pH ~ 10), EDTA, and NaCl lysis buffer containing sodium sarcosinate can be used as well as triton X-100 [79–81, 83, 84, 88]. DMSO also could be included into lysis buffers [79, 80, 83, 88]. Also commercial kits and coated microscopic slides are available for neutral and alkaline Comet assay [77]. Next, agarose gel electrophoresis is carried out in direct electrical current under low voltage. For DNA staining, ethidium bromide, propidium iodide, DAPI, acridine orange [72], or SYBR staining [77, 83–85] can be used. It has been proven that the comets also can be stained with a permanent silver stain [89].

The characteristic comet-like image then can be observed in cells with DNA strand breaks and nucleus fragmentation (Fig. 4). The comet consists of a head and a tail representing different DNA structures. The head contains the nuclear core with macromolecules and unfragmented DNA and the tail consists predominantly of singlestranded DNA. The size of the tail shows the level of DNA damage associated with cell damage. The appearance of the tail can be induced by both necrosis and apoptosis [74, 90], but a characteristic for the

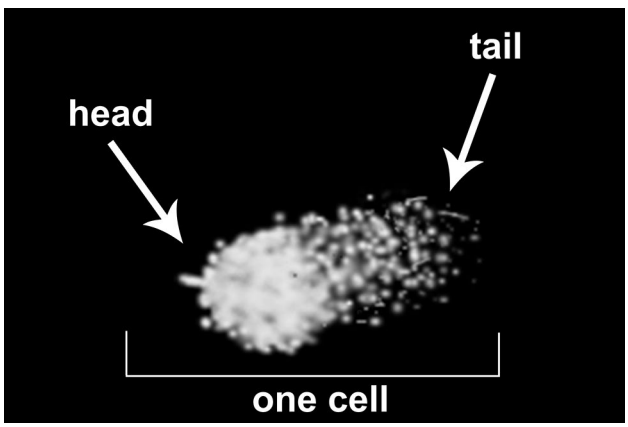

Fig. 3 Comet assay. The comet originates from a single cell after gel electrophoresis

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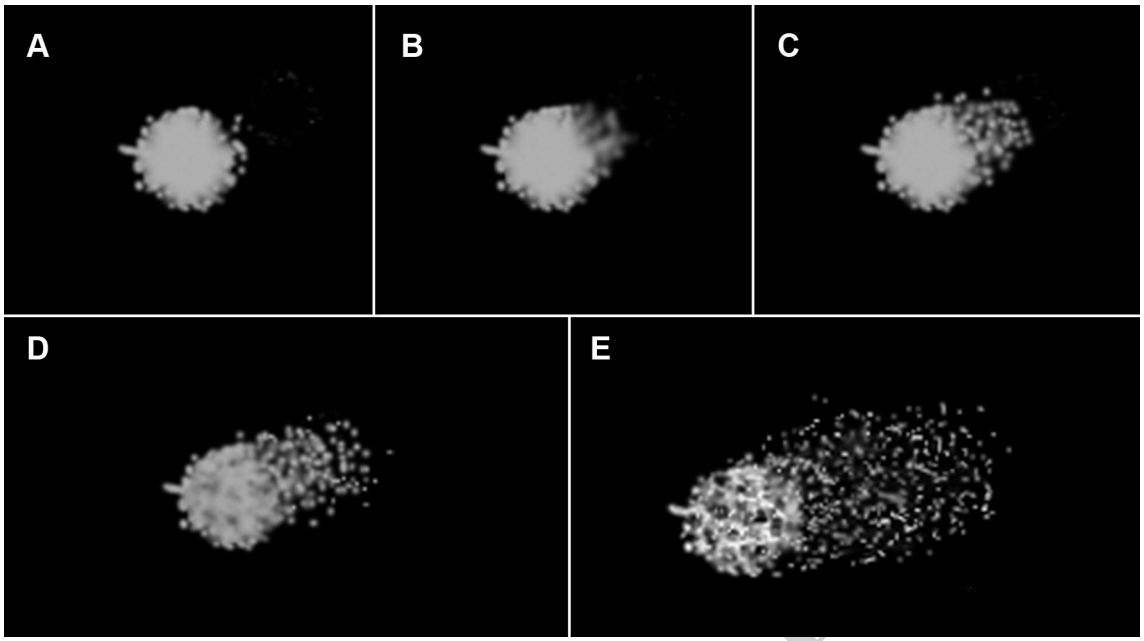

Fig. 4 Visual scoring of comets. Comets are classified into five categories according to the DNA damage: **a** class 0—no or very low damage, **b** low damage, **c** medium damage, **d** long DNA migration, **e** apoptotic or necrotic DNA migration

shape of a comet in apoptotic cells is that most of the DNA moves into the comet's tail [91].

Because the evaluation of these comets can be problematic, it is mostly performed using special software for the visual comet scoring [92]. The scoring is based on measurement as to the magnitude of the comet core and the tail, quantification of fluorescent signal in the core and the tail, and other parameters. The comets are then categorized into groups (Fig. 4) according to the level of DNA damage [68, 93].

Comet assay is a very useful method for detecting DNA strand breaks. It is inexpensive and rapid, with no need for special laboratory equipment when following the protocol by Singh et al. [76]. A disadvantage is that the standard experimental protocols do not allow distinguishing between genotoxicity and early apoptosis [94]. Moreover, comet assay is the only method useful for detecting late stage apoptosis [83, 94]. Therefore, comet assay ought to be used as an additional tool for apoptosis detection [14].

TUNEL assay

The other method based on detection of apoptotic DNA fragmentation is the Terminal deoxynUcleotidyl transferase Nick-End Labeling (TUNEL) technique. It was designed in 1992 by Gorczyca et al. [57] and Gavrieli et al. [53] independently. The key role in the TUNEL assay is played by an endonuclease, in particular terminal deoxynucleotidyl transferase (TdT), catalyzing the attachment of a modified analogue of deoxynucleotides (dUTPs) to the free -OH terminus of the DNA strand breaks [95]. These dUTPs are labeled using various markers that either allow for the detection of DNA strand breaks directly or are able to interact with one or more other detectable markers.

The main workflow of the assay consists of cultivating and harvesting the cells, fixing and permeabilizing cells to allow penetration of the TUNEL reaction reagents into the nucleus, binding of labeled dUTPs onto the -OH moieties of fragmented DNA using TdT, and visualization of the labeled dUTPs. Depending upon the label, the visualization may be fluorescent (most commonly) or enzymatic.

As described also for the DNA ladder assay, the tested cells are cultivated under defined conditions with a specific chemical substance of interest. After cultivation, the cells are fixed using up to 4% formaldehyde [30, 57, 59, 82, 95–98] to prevent leakage of the DNA fragments during the repeated rinsing that is necessary for properly carrying out the TUNEL assay. The cells are then treated in 70% ethanol [57, 59, 95] in order to permeabilize the cells. The permeabilization is necessary for penetration of the TUNEL enzyme TdT into cell nuclei. Various solutions are used to produce the proper functioning of TdT, which catalyzes the incorporation of labeled dUTPs into the DNA strand breaks. In addition to labeled dUTPs, these solutions usually contain sodium or potassium cacodylate [53, 57, 59, 95, 99], cobalt chloride [53, 59, 99], and bovine serum albumin [53, 57, 59, 95, 99]. Some authors also have reported that dithiothreitol [57, 99] can be beneficial for TUNEL reaction.

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Two main dUTPs labeling strategies are used in the TUNEL assay: direct labeling and labeling using bromodeoxyuridine (BrdU), which is a thymidine analogue. The main difference between direct labeling and labeling using BrdU is that in the first case the label can be detected directly. When using BrdU labeling, it is necessary to use an antibody system. The labeling strategies are depicted in Fig. 5.

Direct labeling of dNTPs by fluorescein is a common labeling strategy [59, 100]. In the two original research studies on TUNEL assay, the labeling of dNTPs was performed using biotin, a protein with an affinity for avidin [53, 57]. The paper by Gorczyca et al. [57] describes fluorescently labeled avidin allowing the detection of DNA strand breaks. Gavrieli et al. [53], meanwhile, presented the use of peroxidase-labeled avidin allowing colorimetric detection of DNA strand breaks after adding a specific substrate. Labeling of dNTPs using digoxigenin also has been used in the direct labeling strategy [99, 101]. For BrdU labeling of dNTPs, it is necessary to use anti-BrdU antibodies. These proteins can be labeled by FITC, [59, 95, 102] or by Alexa Fluor™ 488 [103, 104]. In addition to the most widely used BrdU labeling, labeling using another thymidine analogue, 5′-ethynyl-2-deoxyuridine (EdU), also has been developed [105].

The detection of incorporated EdU is performed using so-called "click" reaction, which is covalent conjugation of the ethynyl group of EdU and fluorescent azide as catalyzed by copper [106]. In contrast to BrdU, this EdU assay is not antibody based. The advantage of using 5′-ethynyl-2-deoxyuridine is that EdU's incorporation does not require disruption of the helical DNA structure, as in the case of BrdU. This is due to the fluorescent azide's small size [107].

As mentioned above, the detection of labeled dUTPs together with DNA strand breaks depends on the chosen label and the researcher's requirements. Although light microscopy can be used after staining with horseradish peroxidase-conjugated avidin–biotin complex together with a colorimetric substrate [53, 101], the fluorescence detection has been used most often together with a number of other techniques, including flow cytometry [59, 95, 100], laser scanning cytometry [59, 95, 99], or fluorescence microscopy [95, 97, 98, 108].

TUNEL assay can be regarded as one of the standard histochemical methods for detecting and quantitating apoptotic cells from cell suspensions, adherent cell lines, and tissues in later stages of programmed cell death [101, 109, 110]. TUNEL assay is an accepted assay for establishing apoptosis in vitro and in situ. When confirmed by other methods, it is a reliable test for apoptosis [111]. In comparison with DNA ladder assay, TUNEL staining is more sensitive because it precedes the appearance of the internucleosomal cleavage of DNA detected on the agarose gel [53]. On the other hand, TUNEL assay is able to detect DNA fragmentation not only in apoptotic cells. It is known that DNA damage appears not only during apoptosis but also is linked to necrosis and is caused by toxic compounds or other insults. DNA damage from other sources can thus cause false positive TUNEL assay results [65]. False positivity has been proven also in cells undergoing active DNA repair [112],

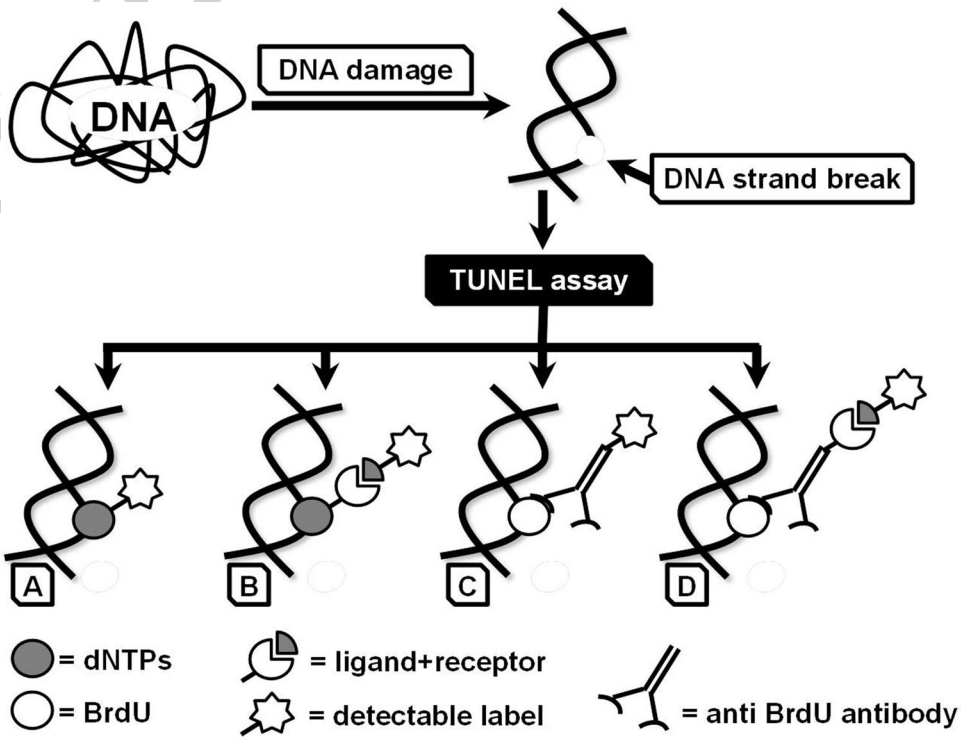

Fig. 5 Options for dNTPs labeling in the TUNEL reaction. Depicted in A and B are direct labeling options, which are: direct binding of a label onto dNTPs (*a*) and binding of the receptor onto dNTPs (*b*). This receptor is able to interact with the ligand, which is fluorescently or enzymatically labeled and allows the detection of DNA strand breaks. Depicted in c and d are labeling possibilities using BrdU: direct binding of BrdU, which interacts with anti-BrdU antibodies labeled with a detectable label (*c*); and binding of BrdU (*d*), which interacts with anti-BrdU antibodies that are themselves labeled with a receptor able to interact with the ligand. That ligand is fluorescently or enzymatically labeled and allows the detection of DNA strand breaks

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in cells undergoing autolysis post mortem [99], and in the degenerating cells appearing in the neonatal brain during the development of acute myocardial infarction [113]. Because TUNEL assay performed in situ is not a method specific only for apoptotic DNA fragmentation [99], it is necessary, as in the case of DNA ladder assay, to compare the results of TUNEL assay also with those from another method.

Conclusion

Apoptosis is a complex process responsible for removing damaged cells from living organisms, and it is connected with characteristic morphological and biochemical changes of the cells. DNA fragmentation occurs during later stages of the apoptotic process. The methods most commonly used for detecting DNA fragmentation are DNA ladder assay, comet assay, and TUNEL assay. These methods are relatively inexpensive and easy to perform. On the other hand, they also can have some limitations, including false positivity. In detecting apoptosis, TUNEL assay is the most sensitive because it is able to detect apoptosis at the phase preceding appearance of the internucleosomal DNA cleavage detected as the DNA ladder and precedes also the shrinkage and destruction of cell nucleus detected using comet assay. All these methods are very useful in apoptosis detection and characterization, but it is appropriate to complement their results using additional methods based on different principles.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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