Preface

Detection and analysis of DNA damage is of critical importance in a variety of biological disciplines studying apoptosis, cell cycle and cell division, carcinogenesis, tumor growth, embryogenesis and aging, neurodegenerative and heart diseases, anticancer drug development, environmental and radiobiological research, and others.

Individual cells within the same tissue or in cell culture may vary in the extent of their DNA damage and, consequently, can display different reactions to it. These differences between individual cells in the same cell population are detected using *in situ* approaches.

In situ is a Latin term meaning "on site" or "in place." It is used to denote the processes occurring or detected in their place of origin. In molecular and cell biology this usually refers to undisrupted mounted cells or tissue sections. In that meaning "*in situ*" is used as part of the terms "*in situ* PCR," "*in situ* transcription," "*in situ* hybridization," "*in situ* end labeling," and "*in situ* ligation." Sometimes the "*in situ*" term is applied at the subcellular level to cells disrupted in the process of analysis, for example, in the detection of specific sequences in chromosomes using fluorescent *in situ* hybridization (FISH). Historically, the term was used primarily in methods dealing with nucleic acids.

In situ methods for the detection of DNA damage can label DNA alterations directly in tissues and individual cells. They differ from the biochemical methods, which label DNA damage in bulk DNA samples obtained after tissue homogenization and cell disruption. *In situ* methods are advantageous in the analysis of heterogeneous cellular populations and in assessing the dynamics and morphological distribution of cellular reactions to various factors. Their attractive features include single-cell detection level, potential to co-localize DNA damage and cellular proteins, ability to use cellular morphology to verify cellular phenomena, small sample size, and, in many cases, the simplicity of performance. The importance of *in situ* approaches is further increased by the conclusion of the human genome mapping, which has put a new emphasis on co-localization of cellular phenomena with various cellular proteins.

Although other volumes dealing with the detection of DNA damage detection have been published, they primarily described biochemical methods

to study DNA alterations. In Situ *Detection of DNA Damage* is the first publication solely dedicated to the *in situ* format. The term "*in situ*" incorporates analysis of both tissue sections and individual cells. The volume brings together all major *in situ* techniques developed to study DNA damage and apoptosis. It also expands the utility of the presented methods by showing how approaches originally designed to label apoptotic cells can be used for DNA damage analysis (and vice versa). It includes many new cutting-edge protocols that have become possible as a result of the significant progress occurring in the field during the last five years.

In Situ *Detection of DNA Damage* is divided into six sections. Each of the major methods to study DNA damage *in situ* is discussed in detail in a separate section consisting of two to six chapters. The first two chapters in Sections 1–4 are theoretical and technical reviews summarizing accumulated information about the techniques described. The remaining chapters in each section concentrate on useful applications.

Sections 1–4 each discuss a single technique or a group of closely related techniques. The complete set of enzymatic approaches to study DNA breaks *in situ* is presented. These techniques were originally introduced to solve a utilitarian task of labeling apoptotic cells and therefore require detailed analysis from the DNA damage detection point of view. The techniques include those designed to detect specific single- and double-stranded DNA breaks in tissue sections using terminal transferase (Chapters 1–6), DNA polymerase I or its Klenow fragment (Chapters 7–9), T4 DNA ligase and T4 polynucleotide kinase (Chapters 10–12). Chapters 13–16 present methods for detection of DNA breaks in agarose-trapped cells and describe the comet assay and related techniques. Detection of modified bases and apurinic/apyrimidinic sites in tissue sections is discussed in Chapters 17 and 18. Chapters 19, 21, and 22 deal with such markers of DNA damage and apoptosis as poly (ADP-ribose) polymerase, p53, and active caspases. In recent years, instrumental techniques for studying DNA damage in tissue sections, single cells, and in vivo have grown significantly in sophistication and power. The novel instrumental techniques presented here describe *in situ* applications of both flow and laser-scanning cytometry for analysis of DNA strand breaks and apoptosis (Chapter 6) and the use of ultrasound for in vivo and *in situ* detection of apoptotic DNA damage (Chapter 20).

In Situ *Detection of DNA Damage* is a comprehensive source of information on every method described. It contains technical reviews discussing specificity, sensitivity, advantages, and limitations of the described techniques in comparison with alternative approaches. Different *in situ* approaches are reviewed with emphasis on their relative merits and shortcomings.

In Situ *Detection of DNA Damage* can be equally useful for both novice scientists and experienced researchers. For a scientist new to the area of *in situ* DNA damage detection, the book will help to select and use the technique most suitable for his/her specific field of study. Detailed explanation of the concepts underlying the methods and the types of DNA damage they label will render it easy for the reader to understand the possible pitfalls in each technique described and to properly interpret experimental results. Experienced researchers actively working in the field will find the book useful because it describes the new approaches, with each method being presented and discussed in greater detail than is generally found in the research literature. Since it provides deeper insight into the types of DNA damage labeled by current apoptosis detection techniques, it is also a helpful resource for molecular scientists studying apoptotic cell death.

Researchers in the fields of cell biology, molecular biology, embryology, toxicology, radiobiology, experimental and clinical pathology, oncology, experimental pharmacology, drug design, and environmental science can benefit from the book.

I would like to thank all participating authors, without whom this book would not be possible and especially those who submitted and revised their contributions promptly. I thank Candace Minchew for technical assistance. I am particularly grateful to Professor John Walker for his advice and help in the review process.

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TUNEL Assay

An Overview of Techniques

Deryk T. Loo

1. Introduction

The study of DNA damage holds a wide interest within both basic and applied fields of research. Elucidating the mechanisms involved in the generation of DNA damage, and the consequences of this damage, will have an enormous impact on multiple fields of scientific research and will ultimately lead to a better understanding of human disease. One of the most widely used methods for detecting DNA damage *in situ* is TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining ***(1)***. TUNEL staining was initially described as a method for staining cells that have undergone programmed cell death, or apoptosis, and exhibit the biochemical hallmark of apoptosis—internucleosomal DNA fragmentation ***(2–6)***. TUNEL staining relies on the ability of the enzyme terminal deoxynucleotidyl transferase to incorporate labeled dUTP into free 3'-hydroxyl termini generated by the fragmentation of genomic DNA into low molecular weight double-stranded DNA and high molecular weight single stranded DNA ***(1)***. While TUNEL staining has nearly universally been adopted as the method of choice for detecting apoptosis *in situ*, it should be recognized that TUNEL staining is not limited to the detection of apoptotic cells. TUNEL staining may also be used to detect DNA damage associated with non-apoptotic events such as necrotic cell death induced by exposure to toxic compounds and other insults ***(7)***, and TUNEL staining has also been reported to stain cells undergoing active DNA repair ***(8)***. Therefore TUNEL staining may be considered generally as a method for the detection of DNA damage (DNA fragmentation), and under the appropriate circumstances, more specifically as a method for identifying apoptotic cells.

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The goal of this chapter is to provide the reader with step-by-step protocols for *in situ* TUNEL staining of nuclear DNA fragmentation in both cultured cells and tissue sections. These basic TUNEL protocols have been used on a wide variety of cell types and tissues with success. Methods for both colorimetric and fluorescent staining of cultured cells and tissues is presented thereby allowing investigators to utilize the TUNEL staining procedure with a minimal investment in laboratory reagents and equipment. In addition, methods to modify and optimize the basic protocols, as well as troubleshooting and control conditions, are provided in the **Notes** and **Troubleshooting** sections.

2. Materials

2.1. Cultured Cells

1. Phosphate-buffered saline (PBS), pH 7.4.
2. 2% buffered formaldehyde: dilute high quality formaldehyde (v/v) in PBS prior to use.
3. 70% ethanol
4. TdT equilibration buffer: 2.5 m*M* Tris-HCl (pH 6.6), 0.2 *M* potassium cacodylate, 2.5 m*M* $CoCl_2$, 0.25 mg/mL bovine serum albumin (BSA). Aliquots may be stored at –20°C for several months.
5. TdT reaction buffer: TdT equilibration buffer containing 0.5 U/µL of TdT enzyme and 40 pmol/µL biotinylated-dUTP (Roche Diagnostics Corp.; Indianapolis, IN). Prepare fresh from stock solutions prior to use.
6. TdT staining buffer: 4× saline-sodium citrate (0.6 *M* NaCl, 60 m*M* sodium citrate), 2.5 µg/mL fluorescein isothiocyanate-conjugated avidin (Amersham Pharmacia Biotech, Inc.; Piscataway, NJ), 0.1% Triton X-100, and 1% BSA. Prepare fresh from stock solutions prior to use.
7. Hoechst 33342 counterstain: 2 µg/mL in PBS (Molecular Probes; Eugene, OR). Stock solution may be stored at 4°C in the dark for several weeks.
8. Vectashield antifade mounting medium (Vector Laboratories, Burlingame, CA).

2.2. Tissue Sections

1. Phosphate-buffered saline (PBS), pH 7.4.
2. 4% buffered formaldehyde: dilute high quality formaldehyde (v/v) in PBS prior to use.
3. 20 µg/mL proteinase K (Roche Diagnostics Corp.). Stock solution may be stored at –20°C for several months.
4. 95, 90, 80, and 70% ethanol in Coplin jars
5. 2% hydrogen peroxide . Prepare fresh from hydrogen peroxide reagent stock prior to use.
6. 2% BSA solution: 2% BSA (w/v) dissolved in PBS and passed through a 0.45 µm filter. Sterile stock solution may be stored at 4°C for several weeks.
7. 2× SSC buffer: 300 m*M* NaCl, 30 m*M* sodium citrate. Stock solution may be stored at room temperature for several months.

8. TdT Equilibration Buffer: 2.5 m*M* Tris-HCl (pH 6.6), 0.2 *M* potassium cacodylate, 2.5 m*M* $CoCl_2$, 0.25 mg/mL BSA. Prepare from stock solutions. Aliquots may be stored at –20°C for several months.
9. TdT Reaction Buffer: TdT Equilibration Buffer containing 0.5 U/µL of TdT enzyme and 40 pmol/µL biotinylated-dUTP (Roche Diagnostics Corp.). Prepare fresh from stock solutions prior to use.
10. Vectastain ABC-peroxidase stock solution (Vector Laboratories, Burlingame, CA).
11. 3,3'-Diaminobenzidine (DAB) staining solution (Vector Laboratories).
12. TdT staining buffer: 4× saline-sodium citrate (0.6 *M* NaCl, 60 m*M* sodium citrate), 2.5 µg/mL fluorescein isothiocyanate-conjugated avidin (Amersham Pharmacia Biotech, Inc.), 0.1% Triton X-100, and 1% BSA. Prepare fresh from stock solutions prior to use.
13. Hematoxylin counterstain (Sigma-Aldrich; St. Louis, MO).
14. Hoechst 33342 counterstain: 2 µg/mL in PBS (Molecular Probes). Stock solution may be stored at 4°C in the dark for several weeks.
15. Vectashield antifade mounting medium (Vector Laboratories).

3. Methods (*see* Notes 1 and 2).

A flowchart of the general protocol for TUNEL staining of cells and tissues is shown in **Fig. 1**. Cells or tissues are fixed with formaldehyde then permeabilized with ethanol to allow penetration of the TUNEL reaction reagents into the cell nucleus. Following fixation and washing, incorporation of biotinylated-dUTP onto the 3' ends of fragmented DNA is carried out in a reaction containing terminal deoxynucleotidyl transferase. Depending on the specific needs of the investigator and/or available equipment, the incorporated biotinylated-dUTP may be visualized by *(1)* fluorescence microscopy or FACS analysis following staining with fluorescent-tagged avidin or *(2)* light microscopy following staining with horseradish peroxidase-conjugated avidin-biotin complex in conjunction with a colorimetric substrate (*see* **Notes 3** and **4**).

3.1. Cultured Cells

3.1.1. Suspension Cells

1. Collect cells by centrifugation, wash with PBS, and resuspend cells at a concentration of $1–2 \times 10^7$/mL in PBS. Transfer 100 µL of cell suspension to a V-bottomed 96-well plate.
2. Fix cells by addition of 100 µL of 2% formaldehyde in PBS, pH 7.4 (see **Note 5**). Incubate on ice for 15 min.
3. Collect cells by centrifugation, wash once with 200 µL of PBS, the postfix with 200 µL of 70% ice-cold ethanol. Cells may be stored in 70% ethanol at –20°C for several days.
4. Collect cells by centrifugation and wash twice with 200 µL PBS.
5. Resuspend cells ($1 \times 10^5 – 5 \times 10^5$) in 50 µL of TdT equilibration buffer. Incubate the cell suspension at 37°C for 10 min with occasional gentle mixing.

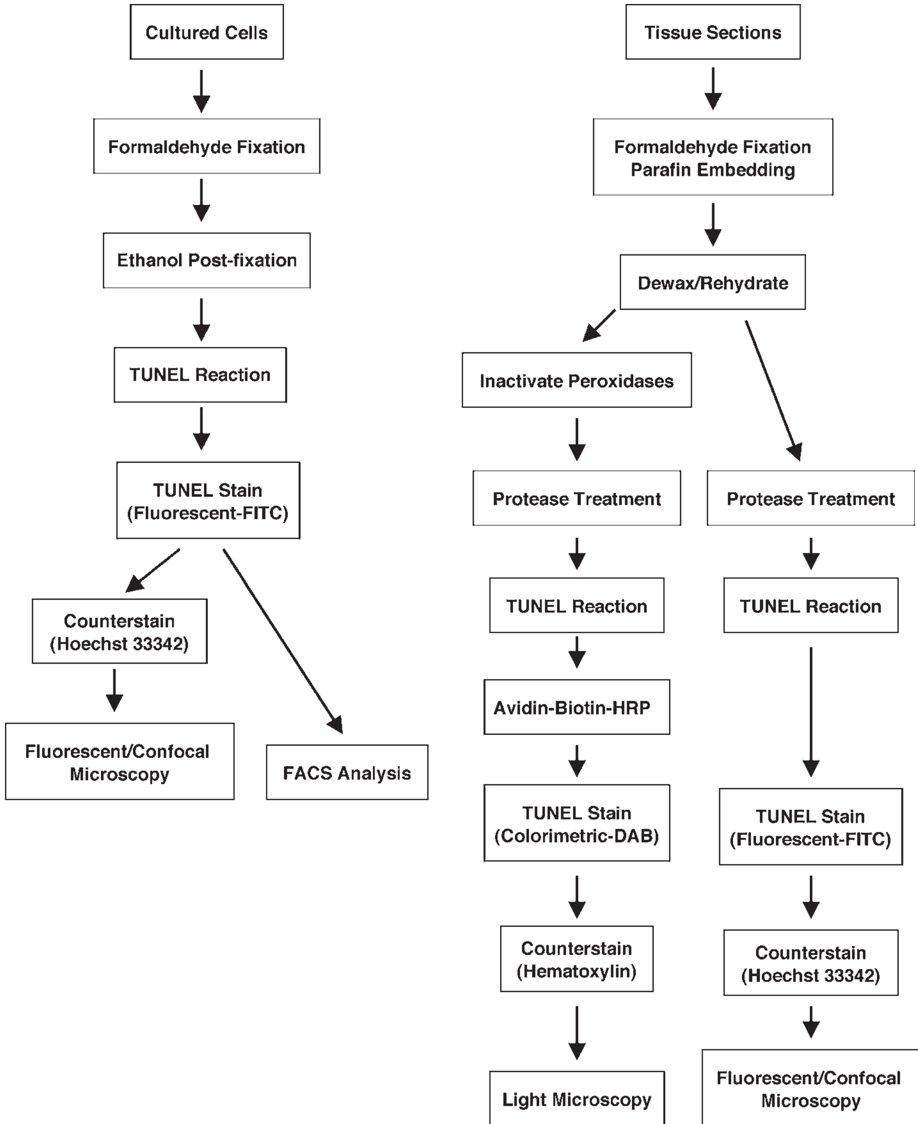

Fig. 1. General flow chart outlining the TUNEL assay protocols described in this chapter for staining cultured cells and tissue sections.

6. Resuspend cells in 50 µL of TdT reaction buffer. Incubate the cell suspension at 37°C for 30 min with occasional gentle mixing.
7. Collect cells by centrifugation and wash with 200 µL PBS.
8. Resuspend the cells in 100 µL of TdT staining buffer. Incubate the cell suspension at room temperature for 30 min in the dark.

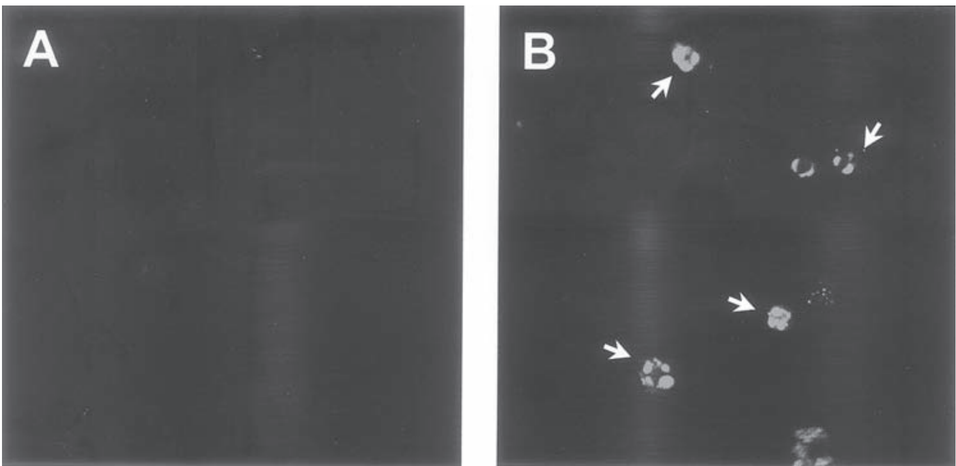

Fig. 2. Confocal micrograph of TUNEL-stained Jurkat T lymphocytes. **(A)** Untreated culture. **(B)** Fas ligand-treated culture undergoing apoptosis. Note the condensed TUNEL-positive chromatin within the nuclei of cells undergoing apoptosis (*see* arrows).

9. Collect cells by centrifugation, wash twice with 200 µL PBS, then resuspend in PBS at 2–8 × 10^6/mL. For fluorescence microscopy attach coverslips using Vectashield antifade mounting medium.
10. Examine cells by fluorescence microscopy, confocal microscopy or flow cytometry. See examples shown in **Fig. 2** and **3**.

3.1.2. Cytospin Preparation of Suspension Cells

TUNEL staining and subsequent fluorescent microscopic or confocal examination of suspension cells may be conveniently carried out on cells attached to glass slides. The cell suspension protocol may be easily modified to accommodate cytospin samples, as described below.

1. Collect cells by centrifugation, wash with PBS, then collect on glass slides pretreated with aqueous 0.01% poly-L-lysine using a cytospin device. Routinely 1 × 10^5 – 5 × 10^5 cells are collected on a single slide.
2. Fix cells by covering with a puddle of 1% formaldehyde in PBS for 15 min.
3. Rinse slides with PBS then transfer to a Coplin jar containing ice-cold 70% ethanol for 1 h. Slides may be stored overnight in 70% ethanol at 4°C.
4. Rinse slides with PBS and pipet 25–50 µL of TdT buffer onto the slides, enough to cover the cells. Incubate the slides in a humidified chamber for 30 min at 37°C. In order to conserve reagents a reduced volume of TdT buffer may be used and carefully covered with a glass coverslip during the incubation. Take care to avoid trapping air bubbles which may lead to staining artifacts.

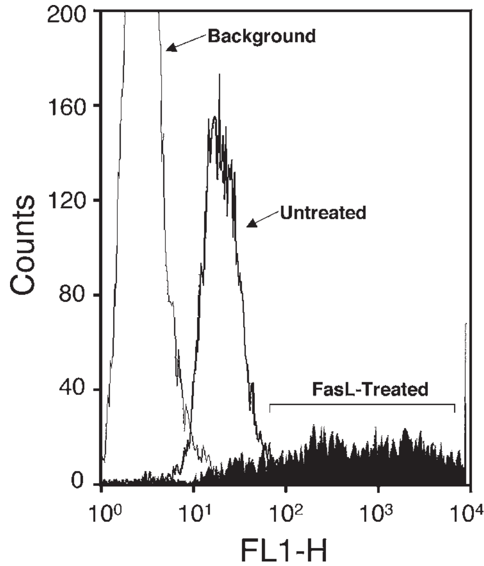

Fig. 3. FACS analysis of TUNEL-stained Jurkat T lymphocytes shown in **Fig. 2**. Note the broad peak of TUNEL-positive fluorescent cells in the FasL-treated sample, indicative of FasL-induced apoptosis.

5. Rinse the slides with PBS then pipet 25–50 µL of TdT staining buffer onto the slides. Incubate for 30 min at room temperature in the dark.
6. Rinse the slides with PBS, air dry, and attach coverslips using Vectashield antifade mounting medium.
7. Examine cells by fluorescence or confocal microscopy.

3.1.3. Adherent Cells

Adherent cells may be cultured on glass chamber slides and processed for TUNEL staining as described above for cytospin-processed cells.

3.2. Tissue Sections

3.2.1. Colorimetric Staining for Light Microscopic Examination

1. Fix tissue samples in 4% formaldehyde in PBS for 24 h and embed in paraffin. Adhere 4–6 µm paraffin sections to glass slides pretreated with 0.01% aqueous solution of poly-L-lysine.
2. Deparaffinize sections by heating the slides for 30 min at 60°C (or 10 min at 70°C) followed by two 5 min incubations in a xylene bath at room temperature in Coplin jars. Rehydrate the tissue samples by transferring the slides through a graded ethanol series: 2 × 3 min 96% ethanol, 1 × 3 min 90% ethanol, 1 × 3 min 80% ethanol, 1 × 3 min 70% ethanol, 1 × 3 min double-distilled water (DDW).

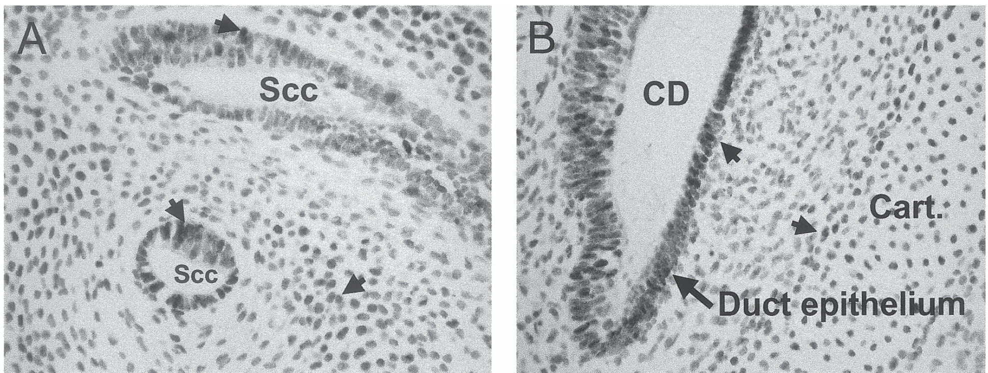

Fig. 4. Micrographs of TUNEL-stained mouse embryo tissue undergoing developmental restructuring (E11–12). **(A)** Semicircular canal area. **(B)** Cochlear duct and primordial cartilage. Apoptotic cells within the duct epithelium and adjacent primordial cartilage exhibiting DNA damage are stained brown (*see* arrowheads). Sections are counter-stained with hematoxylin (blue staining) to identify background TUNEL-negative cells and associated morphology.

3. Carefully blot away excess water and pipet 20 μg/mL proteinase K solution to cover sections. Incubate 15 min at room temperature.
4. Following proteinase K treatment, wash slides 3 × 5 min with DDW.
5. Inactivate endogenous peroxidases by covering sections with 2% hydrogen peroxide for 5 min at room temperature. Wash slides 3 × 5 min with DDW.
6. Carefully blot away excess water then cover sections with TdT equilibration buffer for 10 min at room temperature.
7. Remove TdT equilibration buffer and cover sections with TdT reaction buffer. Incubate slides in a humidified chamber for 30 min at 37°C. In order to conserve reagents a reduced volume of TdT buffer may be carefully covered with a glass coverslip during the incubation. Take care to avoid trapping air bubbles which may lead to staining artifacts.
8. Stop reaction by incubating slides 2 × 10 min in 2×SSC.
9. Rinse slides in PBS then block nonspecific binding by covering tissue sections with 2% BSA solution for 30–60 min at room temperature.
10. Wash slides 2 × 5 min in PBS then incubate in Vectastain ABC-peroxidase solution for 1 h at 37°C.
11. Wash slides 2 × 5 min in PBS then stain with DAB staining solution at room temperature. Monitor color development until desired level of staining is achieved (typically 10–60 min). Stop the reaction by incubating slides in DDW.
12. Lightly counter-stain tissue sections with hematoxylin stain.
13. Cover tissue sections with coverslips using Aqua-Poly/Mount mounting medium.
14. Observe sections under light microscopy. See examples shown in **Fig. 4**.

3.2.2. Fluorescent Staining

1. Follow steps 1–9 outlined above for the colorimetric staining of tissue sections (**Subheading 3.2.1**), omitting the hydrogen peroxide inactivation step.
2. Wash slides 2 × 5 min in PBS then cover tissue sections with TdT staining buffer. Incubate slides at room temperature for 30 min in the dark.
3. Wash slides 2 × 5 min in PBS.
4. Lightly counter-stain sections with hematoxylin, Hoechst 33342 or other appropriate counterstain (*see* **Note 6**).
5. Wash slides with PBS, air dry, and attach coverslips using Vectashield antifade mounting medium.
6. Examine tissue sections by fluorescence or confocal microscopy.

4. Notes

1. The protocols outlined here represent one method, together with several variations, that have been used successfully for TUNEL staining of a broad variety of cultured cells and tissues. However, specific cell types and tissues may require modification or optimization of the staining conditions to obtain successful results. Two of the steps that often require optimization are formaldehyde fixation and PK treatment. A lack of observed TUNEL staining in the positive control sample may result from over-fixation ***(9)***. This result may be remedied by using shorter fixation times and/or reducing the formaldehyde concentration. Additionally, over-treatment with PK may cause a loss of TUNEL staining in the positive control samples. Positive staining in the negative control sample may also result from inadequate inactivation of endogenous peroxidase activity (colorimetric detection), or non-specific binding of the FITC-conjugated avidin reagent (fluorescent detection). In the case of colorimetric detection ensure that the H_2O_2 reagent is fresh. Increasing the H_2O_2 concentration to 5% may also help to reduce the endogenous peroxidase activity. In the case of fluorescent detection, increasing the BSA concentration to 2–5% in the TdT staining buffer and/or reducing the FITC-conjugated avidin concentration may reduce the background fluorescence. It is important to optimize the staining conditions empirically with the control samples prior to examining and interpreting data from valuable experimental samples.
2. While this chapter provide the investigator with a step-by-step method for TUNEL staining that requires a modest amount of reagents, it should be noted that several TUNEL staining kits are now commercially available (R&D Systems, Inc., Minneapolis, MN; Roche Diagnostics Corp., Indianapolis, IN). Commercial kits may be appropriate under certain circumstances, such as for use by the casual or infrequent user or for use in a controlled clinical setting.
3. It is advisable to include control samples with each staining experiment to facilitate interpretation of the staining results. As a positive control, treat cells and tissues with DNAse I (1 µg/mL in 30 m*M* Tris-HCl (pH 7.2), 140 m*M* potassium cacodylate, 4 m*M* $MgCl_2$, 0.1 m*M* DTT) for 10 min at room temperature. Follow-

ing DNase I treatment, wash samples 3 × 2 min in DDW then proceed with TUNEL staining. As a negative control, omit the TdT enzyme from the TdT reaction buffer.

4. Immunohistochemical staining for cell surface or intracellular antigens may be performed simultaneously with TUNEL staining using colorimetric, fluorescent, or a combination of colorimetric and fluorescent detection systems. Careful selection of the detecting reagents will enable simultaneous two color staining of cells and tissue sections that can be observed microscopically, and in the case of suspension cell cultures also by FACS analysis. The reader is encouraged to consult the primary literature to obtain further information on specific systems of interest.
5. Formaldehyde-fixed cell culture samples have been successfully stored in 70% ethanol at –20°C for several weeks prior to TUNEL staining. The suitability of prolonged storage should be determined empirically for the individual culture system employed.
6. Hoechst 33342 binds to DNA and serves as a nuclear stain. Combining Hoechst 33342 staining with TUNEL staining allows one to compare TUNEL-positive nuclei with surrounding normal nuclei and observe changes in nuclear size and morphology. Hoechst 33342 also serves as a counterstain, allowing for the visualization of anatomical structures in both TUNEL-positive and TUNEL-negative cells in cultured cells and tissues ***(9)***.

Acknowledgments

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