

TUNEL Assay

An Overview of Techniques

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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.

From: *Methods in Molecular Biology*, vol. 203: *In Situ Detection of DNA Damage: Methods and Protocols*
Edited by: V. V. Didenko © Humana Press Inc., Totowa, NJ

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 mM Tris-HCl (pH 6.6), 0.2 M potassium cacodylate, 2.5 mM CoCl₂, 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 mM 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 mM NaCl, 30 mM sodium citrate. Stock solution may be stored at room temperature for several months.

8. TdT Equilibration Buffer: 2.5 mM Tris-HCl (pH 6.6), 0.2 M potassium cacodylate, 2.5 mM CoCl₂, 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 mM 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.

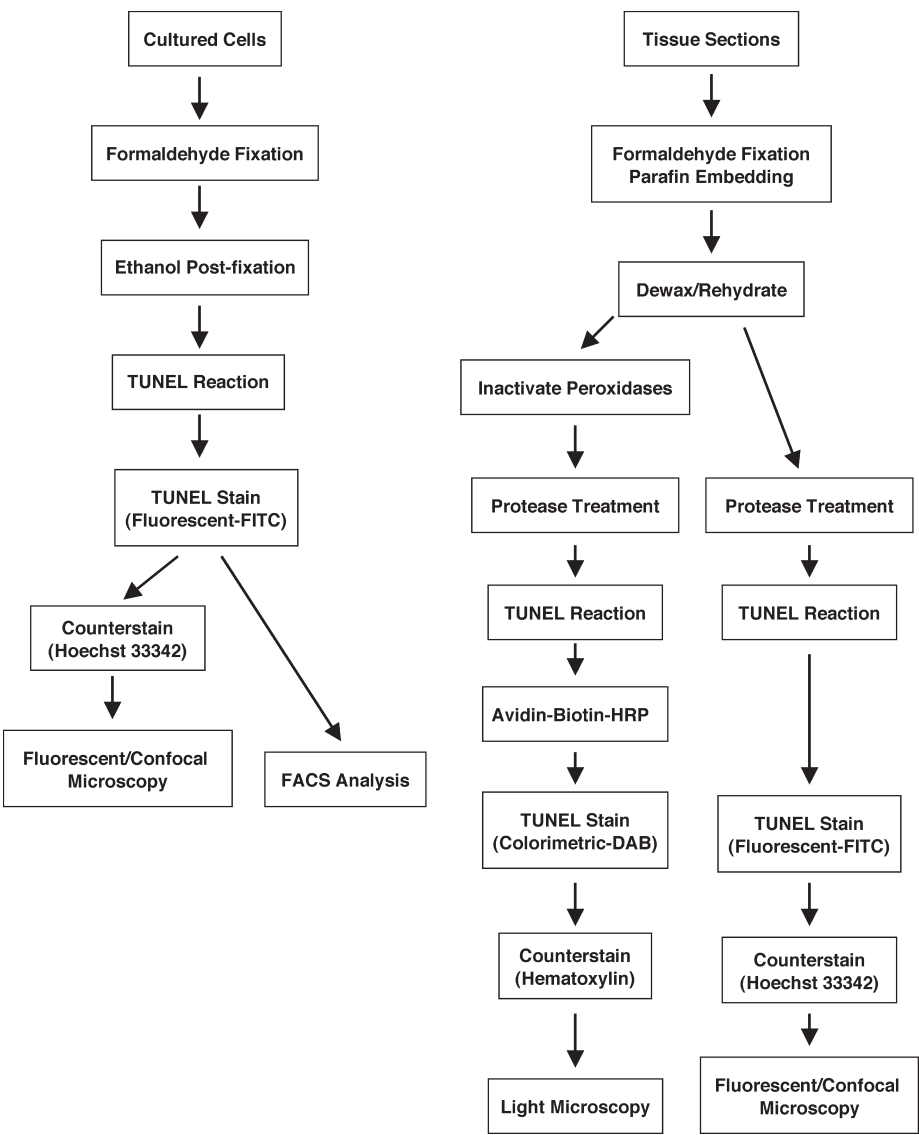


Fig. 1. General flow chart outlining the TUNEL assay protocols described in this chapter for staining cultured cells and tissue sections.

- 6. Resuspend cells in 50 μ L 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 μ L PBS.
- 8. Resuspend the cells in 100 μ L of TdT staining buffer. Incubate the cell suspension at room temperature for 30 min in the dark.

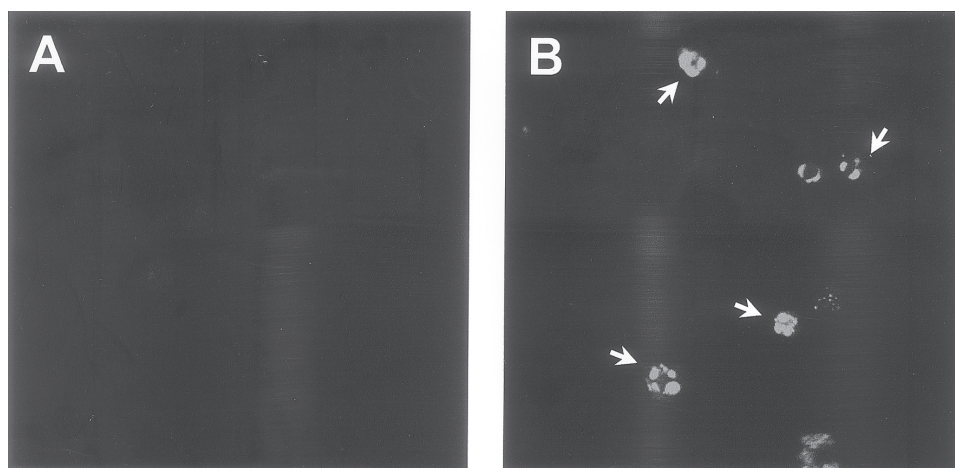


Fig. 2. Confocal micrograph of TUNEL-stained Jurkat T lymphocytes. **(A)** Untreated culture. **(B)** Fas ligand-treated culture undergoing apoptosis. Note the condensed TUNEL-positive chromatin within the nuclei of cells undergoing apoptosis (see arrows).

9. Collect cells by centrifugation, wash twice with 200 μL PBS, then resuspend in PBS at $2\text{--}8 \times 10^6/\text{mL}$. 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 $1 \times 10^5 - 5 \times 10^5$ 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.

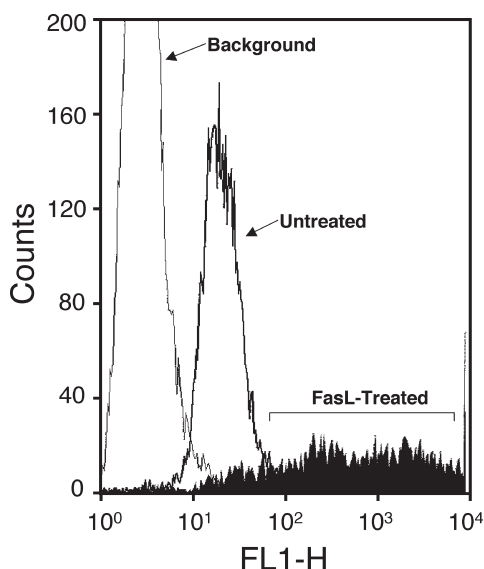


Fig. 3. FACS analysis of TUNEL-stained Jurkat T lymphocytes shown in **Fig. 2**. Note the broad peak of TUNEL-positive fluorescent cells in the FasL-treated sample, indicative of FasL-induced apoptosis.

5. Rinse the slides with PBS then pipet 25–50 μ L 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 cytopsin-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 μ m 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 \times 3 min 96% ethanol, 1 \times 3 min 90% ethanol, 1 \times 3 min 80% ethanol, 1 \times 3 min 70% ethanol, 1 \times 3 min double-distilled water (DDW).

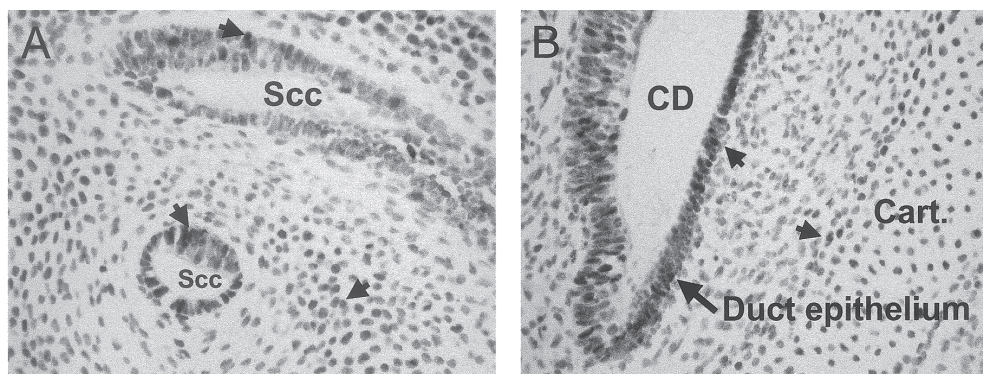


Fig. 4. Micrographs of TUNEL-stained mouse embryo tissue undergoing developmental restructuring (E11–12). **(A)** Semicircular canal area. **(B)** Cochlear duct and primordial cartilage. Apoptotic cells within the duct epithelium and adjacent primordial cartilage exhibiting DNA damage are stained brown (*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 $\mu\text{g/mL}$ 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 $2\times\text{SSC}$.
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 $\mu\text{g}/\text{mL}$ in 30 mM Tris-HCl (pH 7.2), 140 mM potassium cacodylate, 4 mM MgCl_2 , 0.1 mM 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

The author wishes to thank Dr. Donna Dambach for providing the image of the TUNEL-stained tissue sections, Derek Hewgill for expert assistance with confocal microscopy and FACS analysis, Jill Rillema for critical discussion, and Fiona Apple.

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<http://www.springer.com/978-0-89603-952-0>

In Situ Detection of DNA Damage

Methods and Protocols

Didenko, V.V. (Ed.)

2002, XVII, 313 p. 164 illus., 31 illus. in color.,

Hardcover

ISBN: 978-0-89603-952-0

A product of Humana Press